UNIVERSITY OF ALBERTA

# Changes in carbon metabolism alter spontaneous mutation rates in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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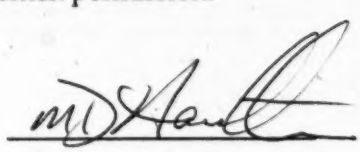

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**Abstract**

Spontaneous mutagenesis is the process that leads to the generation of natural variation. I have demonstrated that the spontaneous mutation rate is subject to regulation by carbon metabolism in response to changes in carbon source. This phenomenon was demonstrated in two yeast species: *Saccharomyces cerevisiae*, which is commonly employed in mutagenesis studies; and *Kluyveromyces lactis*, which has a simplified carbon regulatory system that is more amenable to genetic analysis.

*Saccharomyces cerevisiae* cells grown in ethanol cultures have a 9-fold lower spontaneous mutation rate than cells grown in glucose. *Kluyveromyces lactis* cells exhibit the reverse effect, with a 30-fold enhancement of mutation rate in ethanol-grown cultures relative to glucose cultures. In both species, this phenomenon is under regulatory control by *SNF1* and *MIG1*. Additional strains have been identified in *Saccharomyces cerevisiae* that do not exhibit the carbon source dependent variation in spontaneous mutation rate.

Interactions between DNA repair mutants and carbon source indicate that lesions are preferentially channeled to specific DNA repair pathways when a non-glucose carbon source is present. Recombination-deficient strains grown on ethanol have no detectable spontaneous mutation occurring. A similar lack of spontaneous mutation is observed when nucleotide excision repair mutants are grown on glycerol. Inactivation of *REV3* blocks the majority of spontaneous mutation observed on glucose or glycerol. The change in spontaneous mutation rate is accompanied by changes in the response to UV in *Kluyveromyces lactis*. Cells grown on ethanol have a reduced UV survival and an

attenuated UV-induced revertant yield. This sensitivity to UV is suppressed by deletion of the *mig1* gene.

These results have been incorporated into a model of the interaction between DNA metabolism and carbon metabolism. Carbon metabolism, via the *SNF1* gene product and the *MIG1* gene product, influences the ability of the cell to either recognize or repair DNA damage. This effect appears to be transmitted through a novel, uncharacterized gene *CSM1* (**c**arbon regulation of **s**pontaneous **m**utation). Specific DNA repair pathways appear to be deactivated when non-glucose carbon sources are employed. All of the observed variation in spontaneous mutation rate is dependent on the Rev3/7 DNA polymerase.

My results show that the environment can have very large effects upon spontaneous mutation rates and DNA repair in general. These observations may affect how future research approaches the study of toxin sensitivity, cancer biogenesis, and evolution.

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## Abbreviations

| | |
|---|---|
| CV | Coefficient of variation |
| STRE | Stress responsive element |
| DNA | Deoxyribonucleic acid |
| PCR | Polymerase chain reaction |

## Introduction

Spontaneous mutagenesis is the process by which cells acquire changes in their genetic information. As cells grow and multiply, their genetic information is subject to a variety of challenges and processes which all contain a finite chance of error. The occurrence of errors, or mutations, in the genetic information of a cell can generate new gene variants. This variation is observed at the organism level as the appearance of novel phenotypes in succeeding generations.

Spontaneous mutagenesis is not just a product of the limitations of enzyme specificity during DNA replication, recombination, and repair. The spontaneous mutation rate is likely to be a characteristic that is itself subject to selection. Genetic heterogeneity, or natural variation, is necessary for the survival of a species in evolution theory. If a given population is too homogenous, then the ability of the population to thrive in a new situation is compromised. However, if the amount of variation is too extreme, then the population will be overloaded with deleterious changes. A balance is needed between these two extreme possibilities. The mutation rate will stabilize at a level that is appropriate for that species. Comparison of the spontaneous mutation rates of many organisms indicates that organisms with similar lifestyles have similar mutation rates (Drake, 1998). Thus, all bacteria have similar rates, as do RNA viruses, DNA viruses and mammals. The rate for each group is different from the others, but is consistent within each grouping. The requirements that create this relationship between phylogeny and mutation rate are not known.

Spontaneous mutations, and their causes, also become an important subject when considered from a medical standpoint. Excluding smoking-related cancers, the majority of cancers do not show any correlation with external causes such as environmental mutagens or genetic predisposition. These cancers appear to be the product of a series of mutational events that culminate in the escape of a cell from the limitations to growth imposed by its normal developmental fate (Nowell, 1993). Theoretical studies have suggested that a halving of the rate of occurrence of these events could delay the onset of most cancers past the current median age of death, effectively eliminating over 80% of the cancer currently being treated (Loeb and Christians, 1996). For example, virus-associated hepatocellular carcinoma has a lag time between viral infection and cancer detection of approximately 40 years. Most infection occurs during childhood, placing the age of onset for the cancer in the late 40's and early 50's. A 2-fold reduction of the rate of mutagenesis could move the age of onset to 85 years and older, well beyond the average life expectancy. Consequently, implementation of methods for lowering the spontaneous mutation rate very likely would increase the average human life span by eliminating early cancer-related deaths. A further extension of life expectancy could be expected if the spontaneous mutation rate theory of aging is even partially correct (Holliday, 1995).

The processes that generate spontaneous mutations create a situation that places the survival of the group (*i.e.* evolution of a species) in conflict with the survival of the individual (*i.e.* cancer or inviability). The balancing of these apparently incompatible results leads to the appropriate spontaneous mutation rate for a given species. The appropriate rate is set in accordance with forces and directives that are not understood. A

low mutation rate would condemn a species to probable extinction under high selective pressure, and conversely, a species with a high mutation rate would be hobbled under stable conditions. Competitive experiments using mutator strains of *E. coli* have shown that mutator strains are favored under selective conditions (Trobner and Piechocki, 1984b), but under stable conditions, even in the absence of competition, additional mutations compensating for the mutator function accumulate in the population (Trobner and Piechocki, 1984a). The most successful species would be one that allows the current conditions to affect its spontaneous mutation rate; in essence, allowing mutation rates to rise for situations requiring rapid change, and subsequently decrease once the surrounding conditions are stable. This hypothesis suggests that the surrounding conditions influence the mutation rate. The hypermutability observed in growth-restricted *E. coli* cells, commonly called adaptive mutation, is one situation where the growth situation directly affects the mutation rate of the population. The growth arrest leads to the depletion of MutL protein and loss of mismatch repair function (Harris *et al.* 1997). The loss of mismatch repair causes the incorporation of replication-associated errors to increase, allowing an increased mutation rate until the cell escapes the growth arrest and can re-establish the low spontaneous mutation rate appropriate to active growth.

The change in mutation rate need not require such a dramatic change in the ability of the cell to grow and, by extension, the ability of the population to adapt to new conditions. It is possible that specific small changes in the environment could be detected by the cell. These environmental triggers would likely be changes that affect some core aspect of the life cycle of a given organism, thus presaging an upcoming

selective pressure. In this study, I have identified the carbon source as an environmental signal that alters the spontaneous mutation rate in yeast populations.

Early Work.

The turn of the century saw the initiation of genetics as a discipline with the rediscovery of Mendel's work (1866) and the independent confirmation by de Vries (1900), von Tschermak (1900), and Correns (1900). The theory of evolution by natural selection described by Darwin (1859) led to the question of how natural variation occurs. The suggestion by Sutton (1902) that chromosomes carried the units of inheritance led to the first investigation of spontaneous mutation. Following from a suggestion by Alfred Sturtevant (see Sturtevant, 1978), Muller and Altenburg (1919) made the first measurement of spontaneous mutation rate by measuring the occurrence of X-linked lethal events in *Drosophila*. They were able to establish a rate of 1 lethal mutation per 53 X-chromosomes per sexual generation, much higher than expected.

The research on spontaneous mutation, mostly in micro-organisms, became focused on resolving the question of whether the events which led to the observed changes in phenotype were a part of the normal cellular processes or induced by the presence of the stimulating condition which allowed identification of the mutants. This work was performed using bacterial cultures that were grown from very few cells to saturation of the growth media, and then challenged with lytic virus. Almost all cultures would generate a few colonies that were resistant to the virus, and the progeny of such

colonies retained the virus-resistant phenotype. D'Herelle (1926) believed that interaction with the virus led to the acquisition of resistance, while Gratia (1921) and Burnet (1929) proposed that the resistant cells were present in the culture prior to the addition of the virus. The primary impediment to resolution of this question was the inability to track the occurrence of the mutations without imposing a selection on the population. The high variability in the frequency of the detected mutants from experiment to experiment prevented discrimination between the opposing hypotheses.

The question was resolved by Luria and Delbruck (1943) when they developed their fluctuation test of spontaneous mutation. Multiple cultures of bacteria are grown independently, and then challenged by virus after growth has ceased. Each culture will generate a number of resistant cells that are able to grow in the presence of virus. They reasoned that if exposure to the virus was an essential part in development of resistance, then every culture should generate similar numbers of resistant cells. On the other hand, if the development of resistance occurred at random during the growth of the culture, then the number of resistant cells present at the end of growth will be much more variable. The results of their experiments conformed to the expectation that the mutation was acquired prior to exposure to the virus. Demerec (1945) and Witkin (1947) subsequently confirmed their work for antibiotic resistance and resistance to X-rays, respectively.

Once spontaneous mutation was established as an ongoing process that occurs during normal growth, investigation of the causes of spontaneous mutation began. Given the ease with which X-rays could induce mutants, environmental X-rays were proposed as a likely source of spontaneous mutations. Careful study of the kinetics of mutagenic

effects of X-rays indicated that the beginning of linearity of X-ray -induced events above the spontaneous background was approximately at 1 gray (von Borstel, 1966). The fact that X-ray induced mutation could be observed at levels as low as 25 centigrays (Spencer and Stern, 1948) was used to establish linearity of radiation-induced mutational damage to one order of magnitude below the spontaneous level (von Borstel, 1966). The spontaneous mutation frequency per generation was approximately equal to that generated by one-tenth of a centigray of ionizing radiation -- four orders of magnitude higher than that which would be caused by the natural background radiation exposure rate per sexual generation of the fruit fly.

In 1937, Demerec demonstrated that different strains of *Drosophila melanogaster* had different spontaneous mutation rates. Ives (1950) discussed possible effects on rates of evolution of *Drosophila* strains with different spontaneous mutation rates. After McClintock discovered mobile elements in the chromosomes of *Zea mays* (McClintock, 1951), Green (1969) sought and found putative mobile elements in *Drosophila melanogaster* that altered the spontaneous mutation rates in a strain-dependent manner. The strain dependent variability observed by Demerec was due to the mobile element activity, not to a cellular process. Though important to the evolution of *Drosophila* populations, hybrid dysgenesis as a source of spontaneous mutation could not be extrapolated to other systems.

It became apparent that DNA metabolism was intimately involved in the process leading to spontaneous mutation. The earliest of the 3 Rs (replication, recombination and repair) to be introduced as a factor in spontaneous mutation was the discovery by Magni and von Borstel (1962) that the spontaneous reversion rate was higher for certain alleles

during meiosis than during mitosis. Magni (1963) ascribed this "meiotic effect" to a higher recombination rate during meiosis, as shown by a higher recombination of outside markers associated with the reversion event. This effect was observed only for frameshift mutations; base substitution mutations did not exhibit the meiotic effect (Magni, 1964; 1969). Atwood (1953) proposed an editing function which could overcome the thermodynamic limitations of simple base pairing during replication, a function which was strongly supported by the work of Speyer (1965; Speyer *et al.*, 1966) and Drake *et al.* (1969) and ultimately confirmed by Brutlag and Kornberg in 1972.

In 1958 Ruth Hill discovered a mutant strain of *E. coli* that was sensitive to ultraviolet radiation. This indicated that the more sensitive strain had lost an ability to repair its DNA, suggesting a genetic component to DNA stability and sensitivity to mutagens. Hanawalt and Haynes (1965) hypothesized that DNA repair mutants should have an increased rate of spontaneous mutation. Subsequently, separate studies with different species showed that mutants which had DNA repair defects have altered spontaneous mutation rates (Böhme, 1967; Jyssum, 1968; Mohn, 1968; von Borstel, 1968; Zakharov et al., 1968). DNA metabolism was firmly established as the source of spontaneous mutation.

## DNA Metabolism and Spontaneous Mutation

The replication and partitioning of DNA to daughter cells makes up a large part of the activity of an actively dividing cell. Replication is highly regulated, both in initiation

and at defined checkpoints throughout the cycle. Once the cell passes Start, the expression of a large number of genes is induced. A number of these assemble into a complex on the DNA that has been termed a replisome (Jazwinski and Edelman, 1984). This complex contains all of the proteins that are necessary for replicating the DNA with high fidelity.

The greatest contributor to replication fidelity is the DNA polymerase itself. The polymerase is responsible for the selection and insertion of the correct base, and the first error-checking activity, a proofreading exonuclease that removes unstable bases from the growing chain. The DNA polymerase enzyme is responsible for selection and insertion of each incoming base and proofreading the nascent base pair. The combination of these actions will incorporate one incorrectly paired base for every $10^6$ base pairs synthesized (Schaaper, 1993).

This level of error is still too high for most organisms, and supplementary repair systems have been incorporated into the replication process, bringing the error rate down to $10^{-10}$ errors per base pair synthesized. Mismatch repair is responsible for the majority of the repair activity that removes the errors produced by the polymerase. Inactivation of mismatch repair in *Saccharomyces cerevisiae* increases spontaneous mutation rates in a locus-specific manner, varying from 25-fold for forward mutation of the *CAN* locus, to 850-fold for reversion of the *hom3-10* locus (Tishkoff *et al.*, 1997a). Mismatch repair appears to recognize single base pair mismatches and frameshifts, and small single-stranded loops (Marsischky *et al.*, 1996).

There are specific types of sequence motifs that are at a particularly high-risk of change during replication. The best known, for their role in the etiology of many

inherited disorders in humans, is microsatellites. These are short sequences, 2-4 base pairs in length, which are repeated many times in tandem. As the replication machinery moves through one of these regions, the newly synthesized strand will contain more or fewer repeats. Relative to base mutation rates, these sites will have addition/deletion mutation rates as much as 80-fold higher in *Saccharomyces cerevisiae*, depending on the number of unit repeats (Tran *et al.*, 1997, Sia *et al.*, 1997). A related motif is short distant repeats that can generate short deletions and duplications. These errors are greatly enhanced in *rad27* or *pol2* mutants, and require *RAD52* function (Tishkoff *et al.*, 1997b; Tran *et al.*, 1995). These structures are also hot spots for spontaneous recombination in yeast (Tran *et al.*, 1997).

The progress of DNA replication is monitored by gene products that assess the completion of replication and the presence of DNA damage at the S/G2 transition (*RAD9*, *MEC1*, and *RAD17*, in *Saccharomyces cerevisiae* (Weinert and Hartwell, 1988)). The absence of the *RAD9* gene causes a 5-fold increase in spontaneous recombination events, both homologous and non-homologous, indicating that recombinogenic damage is not uncommon at the end of replication and suggesting that chromosome breaks may be the lesion that the Rad9 pathway is monitoring (Fasullo *et al.*, 1998).

Exogenous agents such as radiation and reactive chemicals can damage DNA. Reactive compounds in the cells during growth might cause similar damage. If so, the lack of a specific DNA repair activity would increase the likelihood of a mutation since the repair of the damage by the cell would be that much more difficult. This hypothesis has been demonstrated for a variety of DNA repair genes (*cf* Haynes and Kunz, 1981; *cf* Demple and Harrison, 1994; Brychcy and von Borstel, 1977; Prakash, 1981; Ramotar *et*

*al.*, 1991; Leifshitz *et al.*, 1998). The increased spontaneous mutation rate is likely due to repair by an alternate repair pathway, as proposed by Hastings *et al.* (1976). DNA damage that remains unrepaired by an efficient process (for instance nucleotide excision repair of thymidine dimers) would have to be repaired by another repair pathway that is more likely to generate errors during the repair process.

A very large number of endogenous chemicals have been suggested as possible contributors to spontaneous mutation (Soloway and Lequesne, 1980; Talpaert-Borlè, 1987; *cf* Lutz, 1990; Esterbauer *et al.*, 1990; Glatt, 1990; Rossman and Goncharova, 1998). These chemicals include lipid peroxides, acetaldehyde, sugars, S-adenosyl methionine, metal ions, steroids, amino acids, and oxygen radicals. Indeed, treatment of cells with some of these chemicals can cause increases in mutation frequency. However, experiments that have attempted to prove such a relationship directly by the identification of lesions that are unequivocally caused by the chemical under study at doses where the mutagenic effect of the chemical is not detectable have failed. For example, the examination of 8-oxo-dG as a measure of oxidative damage has shown that the amount of damage detected varies with the detection method used and with the DNA isolation method (England *et al.*, 1998). Both steps cause the formation of artifactual 8-oxo-dG, often to levels which would indicate that all of the damage detected in untreated cells is induced during purification and testing of the DNA (Anson *et al.*, 2000).

Detection of DNA lesions at spontaneous levels is extremely difficult. The post-labeling method developed by Randerath *et al.* (1981) has allowed detection of putative lesions (I-compounds) in unmutagenized DNA. The number and amount of these compounds varies with age, nutrition, genotype, and tissue. However, despite their

original hypothesis that these represented pre-mutagenic lesions, the experimental evidence suggests that these compounds are natural modifications that serve to protect DNA. The level of these I-compounds increases in animals kept under dietary restriction, a state that has been shown to be anti-carcinogenic (Zhou *et al.*, 1999). In animals treated with carcinogens, the level of some of these I-compounds declines as the level of carcinogen increases (Vulimiri *et al.*, 1995; Randerath, *et al.*, 1995).

The increased rate of spontaneous mutation that is observed in most strains which are DNA repair deficient strongly suggests that DNA damage is a factor in the development of spontaneous mutation. Almost all of the increase in spontaneous mutation observed in DNA repair mutants in yeast can be eliminated by a second mutation which inactivates polζ (Leifshitz, *et al.*, 1998; Holbeck and Strathern, 1997; Roche *et al.*, 1995; Roche *et al.*, 1994; Halas *et al.*, 1997). This non-essential polymerase is encoded by the *REV3* and *REV7* genes, and has been shown to be required for many aspects of spontaneous and induced mutagenesis. Recent work suggests that much of spontaneous mutation in eukaryotes is caused by a family of DNA repair polymerases which have a higher error rate than replicative DNA polymerases (Johnson *et al.*, 1999; Freidberg and Gerlach, 1999). It is not the identification of the damaged DNA and the removal of the damaged region, but errors incorporated during the resynthesis step that generates the mutation. Removing the mutagenic polymerase (by gene knockout) eliminates the majority of the spontaneous mutation events in *Saccharomyces cerevisiae*.

Direct isolation of mutator mutants has yielded the expected DNA replication mutants and DNA repair mutants (as scored by mutagen sensitivity) (von Borstel *et al.*, 1973; 1993; Nasim and Brychy, 1979). Of 44 tested isolates with altered mutation rates,

27 did not have an associated sensitivity to γ-ray or UV treatment, suggesting that they are not part of the three major DNA repair pathways. Unfortunately, further analysis of these mutants has been limited.

Spontaneous mutation rates are generally discussed as a characteristic of a cell that is relatively constant. I had no reason to question this belief until I used glycerol as a carbon source in one of my early experiments on adaptive mutation prior to this work. A very different rate of mutation was observed, though all conditions of the experiment were the same; only the carbon source was changed. This observation was repeatable.

The only plausible explanation was that the carbon source was affecting the spontaneous mutation rate. This initial observation led to the hypothesis that carbon metabolism and DNA metabolism are linked, and that changes in carbon metabolism could alter some aspect of spontaneous mutation.

## Carbon Metabolism

Carbon metabolism is the central pathway of the yeast cell. Yeast cells are able to utilize a variety of carbon sources, but some carbon sources, notably glucose, are more easily converted into usable energy and materials than others are. A complex regulatory system has evolved which allows preferential use of glucose in mixed carbon cultures. This system is commonly referred to as catabolite repression, however it has become apparent that both inductive and repressing activities occur in response to glucose signaling. I will use the more representative phrase "catabolite regulation" in the

following discussion. The following discussion is drawn from three excellent reviews; Gancedo (1998); Johnston (1999); and Wills (1990).

The primary signal molecule in the establishment of different gene expression patterns is the presence/absence of glucose in the surrounding medium. In the presence of glucose, the genes that are required for the catabolism of different sugars or carbon sources are repressed. When glucose is depleted from the medium, this repression is removed. The presence of other carbon sources induces expression of genes required for the catabolism of that carbon source. The transition from one carbon source to another requires a slowing of the cell cycle as the change in gene expression pattern takes place. This slowing of cell proliferation, called the diauxic shift, appears in growth curves as a plateau between separate logarithmic growth phases.

*Saccharomyces cerevisiae* exhibits the Crabtree effect (Crabtree, 1929), whereby the mitochondria are inactive during growth on "high" concentrations of glucose (>0.2%), and the end products of glycolysis are excreted from the cell as ethanol and carbon dioxide[1]. The regulation of carbon metabolism is controlled by a series of kinases and dephosphorylases. The initial recognition of glucose in the media occurs when glucose transporters and receptors in the cell membrane bind to and transport glucose into the cell.

The primary repressive pathway is initiated by the transport of glucose into the cell by Hxt1 and a coupled phosphorylation by Hxk2. There are three glucose kinases in *Saccharomyces cerevisiae,* but only Hxk2 is essential for gene repression caused by the presence of glucose. The Hxk2 activity inhibits the activity of the Snf1/Snf4/Sip kinase

[1] The discovery of this particular property of yeast has been described as one of the earliest steps toward the establishment of agriculture, and by extension, civilization. (Sauer, 1952)

complex. The loss of Snf1/4 kinase activity leads to underphosphorylation of Mig1, allowing it access to the nucleus. Mig1 is able to target the general transcriptional repressor Ssn6/Tup1 to a large number of genes, preventing their expression. These genes include the transcriptional activators *HAP2/HAP3/HAP4*, required for expression of many mitochondrial proteins and the genes required for gluconeogenesis; *GAL4*, necessary for full expression of the genes required for galactose catabolism; and *MAL63*, which activate genes specific for the catabolism of maltose. A very similar protein, Mig2, also is able to prevent gene expression in a subset of the genes controlled by Mig1, but is regulated by a different, unknown pathway.

The presence of glucose in the surrounding media stimulates the expression of genes in the glycolytic pathway. This induction of glycolytic genes is dependent upon two glucose sensors, Rgt2 and Snf3. In the presence of glucose, the sensors generate a signal that inactivates a transcriptional repressor, Rgt1, which limits expression of several glycolytic genes. The inactivation of Rgt1 requires Grr1, which is associated in the cell with the SCF complex that contains an ubiquitin-conjugating protein, Cdc34 (Kishi *et al.*, 1998). This complex is known to be responsible for protein degradation in several regulatory pathways (Willems *et al.*, 1999).

The initial observation I made that glycerol altered the spontaneous reversion rate in *Saccharomyces cerevisiae* immediately suggested that some aspect of DNA metabolism must be affected by the carbon metabolism. We failed to find any prior publications describing modulation of DNA metabolism in response to signals generated by metabolic and catabolic pathways. Previous work has shown that the genes which code for replication proteins are coordinately regulated by cell-cycle events (Toone *et al.*,

1997), and that a number of genes are induced as part of the cellular response to DNA damage (Ruby and Szostak, 1985; Jelinsky and Samson, 1999; Lowndes and Murguia, 2000). Some DNA repair genes contain STRE (**stress response**) elements, which have been shown to be associated with genes induced in stressed cells (*i.e.* heat shock; Mitchel and Morrison, 1983, 1986).

The investigation of catabolite regulation in *Saccharomyces cerevisiae* has been limited by the redundancy found throughout the system. There are three glucokinases (encoded by *GLK1*, *HXK1*, *HXK2*), three repressor proteins (*MIG1*, *MIG2*, *YER028*; Lutfiyya etal., 1998), five Snf1/4-associated proteins (*SIP1*, *SIP2*, *SIP4*,*GAL83*, *SIP5*; Jiang and Carlson, 1997; Vincent and Carlson,1998; Sanz *et al.*, 2000), and 20 glucose transporters/receptors (Ozcan and Johnston, 1999). The redundancy of the system has placed limits upon the effectiveness of mutant screens, as similar screens performed in different laboratories have not recovered similar patterns of mutants, and some mutant phenotypes disappear in different backgrounds (Trumbly, 1992).

In order to escape the limitations of *Saccharomyces cerevisiae*, the investigation of the effect of carbon regulation was performed in part using the yeast *Kluyveromyces lactis*. This species is a related *Saccharomycetales* yeast and has been shown to have a much simpler carbon regulatory system (Wesolowski-Louvel *et al.*, 1992; Goffrini *et al.*, 1996) which lacks much of the redundancy observed in *S. cerevisiae*. There are four glucose transporters vs. twenty in *Saccharomyces cerevisiae*; one glucose kinase *vs.* three in *S. cerevisiae*; one *MIG1* homolog, *vs.* three in *S. cerevisiae*; and one Snf1/4-associated protein *vs.* five in *S. cerevisae*. The regulatory genes *SNF1*, and *MIG1* are functionally equivalent in interspecies complementation tests between the two yeasts (Cassart *et al.*,

1997; P. Goffrini, personal communication). *K. lactis* is also a petite-negative yeast; it must have functional mitochondrial DNA in order to grow (Clark-Walker and Chen, 1996).

The discovery of a modulating influence of carbon metabolism on spontaneous mutation has presented the opportunity for development of a new area of research into the mechanisms of DNA homeostasis and mutation. In this thesis, I characterize the effects of carbon metabolism on spontaneous and induced mutation, and identify genes involved in regulating DNA metabolism in response to changes in carbon metabolism.

## Methods

Strains.

The strains used in this study are shown in Table 1. The *Kluyveromyces* strains were obtained from Iliana Ferrero's laboratory. The *rag5*, *mig1* and *fog2* genes are the functional equivalent of *hxk2*, *mig1* and *snf1*, respectively, in *S. cerevisiae* (Gancedo, 1998). Strain XV185-14C (and the majority of the *Saccharomyces cerevisiae* strains used in this study) carries several alleles that allow the study of several different mutational events in parallel. The *ade2-1*, *arg4-17* and *lys1-1* mutants are ochre mutants. The *ade2-1* marker can be suppressed by type II tRNA suppressors; the *arg4-17* and *lys1-1* markers are Class I suppressible alleles. The *ade2-1* mutation, which confers a red color to the colony, is included to allow discrimination of locus revertants from suppressor reversions. Locus revertants at *arg4* or *lys1* remain red, while suppressor mutations also suppress the *ade2* phenotype, causing the colony to appear white. The *his1-7* allele is a missense allele which is capable of reverting at many sites in the gene (von Borstel *et al.*, 1998). All types of base substitutions have been observed in a pattern that resembles the *URA3* forward mutation spectrum (Lee *et al.*, 1988). The *ura3-13* allele is a 23-bp duplication that reverts by removal of the duplicated segment.

The repair deficient *Saccharomyces* strains are all derived from strains originally obtained from the Yeast Genetic Stock Center which were previously crossed into the genetic background in use in our laboratory (P. J. Hastings, personal communication).

Cell growth and media.

Two different species of yeast, *Kluyveromyces lactis* and *Saccharomyces cerevisiae,* were utilized during these studies. Their growth requirements are different, entailing separate media formulations. *Saccharomyces cerevisiae* cells were maintained on YEPD plates (2% glucose, 1% yeast extract, 2% peptone). *Kluyveromyces lactis* was maintained on YEPD(K) (2% glucose, 2% yeast extract, 1% peptone). Cultures intended for long term storage were grown in rich media with shaking to saturation; DMSO was added to a final concentration of 7%, and aliquots were frozen at -70° C. Defined media contained 0.67% yeast nitrogen base (Difco or Amersham, filter-sterilized as a 10X concentrate and added following heat sterilization of the agar solution), 2% glucose or other carbon source, and was supplemented with amino acids and bases as shown in Table 2. Specific alterations in the amino acid supplementation were made for specific experiments. These changes are detailed in Table 3. For solid medium, agar was added to 1.5%.

Lassie tests.

Cell concentration of an overnight liquid culture was determined microscopically using a Brightline hemocytometer, and cells from this culture were resuspended in sterile water to a concentration of $5 \times 10^6$ cells/ml. 0.5 mls of this suspension was plated on each of 2-4 plates containing a small amount of an amino acid (limiting) and 2-4 plates lacking the amino acid (dropout). Limiting concentrations for specific selections are shown in Table 3. When *arg4-17* was the selectable marker, the amount of adenine was

reduced in order to permit discrimination of suppressor and non-suppressor alleles (Table 3).

After 5-8 days of growth at 28° C, the number of colonies on each plate was determined, and an average number per plate calculated. The average number of colonies per dropout plate is subtracted from the average number of colonies per limiting plate to generate the "Lassie score".

Compartmentalization tests.

The compartmentalization test has been described in detail by von Borstel *et al.*, 1971. Each experiment required distribution of limiting media to each well of 25 4x6 cell culture plates (600 wells), 1 ml per well. Cells from an overnight liquid culture in YEPD were washed once with sterile water, and total cell number determined by direct count using an haemocytometer (Brightline). The cell suspension was diluted to 80,000 cells/ml, and 25 µl, containing ~2000 cells, was added to each compartment. The plates were incubated at 26° C, 90% humidity, for 10-14 days. 0.5 ml aliquots of an additional cell suspension of $5 \times 10^6$ cells per ml were plated on dropout media to establish the frequency of pre-existing revertants in the plating culture.

The plates were examined for the presence of colonies or papillae that grew out of the background growth. Only the first revertant to appear in a given compartment was counted since many of the revertants release the limiting metabolite to the surrounding culture enabling additional growth of non-revertant cells and additional opportunity for reversion which is not common to all compartments. Wells lacking visible colonies were washed with 1 ml of water to resuspend the cells for counting and verification of non-

revertant phenotype. 10-15 wells were counted by hemocytometer to determine an average total cell growth per compartment. Compartment classifications were verified by spotting the resuspended cells onto defined media lacking the limiting metabolite.

The cells of some strains were difficult to dissociate from the surface of the agar. These cells were released by adding 1.0 ml of 6.0 N HCl, 1.0 M sorbitol. Agar dissolves at pH less than 3.0, which releases the cells for counting. The sorbitol serves as an osmoprotectant, allowing the cells to retain their shape for counting. Cell counts were corrected for the increased volume the dissolved agar lent to the suspension.

The mutation rate is calculated using the $P_o$ method as shown below (taken from von Borstel *et al.*, 1971).

> *Computation of mutation rates:* Let $N$ be the number of compartments in an experiment, and $N_0$ the number of compartments without revertants. From the zeroth term of a Poisson distribution we have
>
> $$e^{-m} = N_0/N, \quad (1)$$
>
> where $m$ equals the average number of mutational events (not mutants) per compartment. Most of these mutational events are due to new mutations arising during the growth of the cells in the limiting medium, but some are due to mutants present in the inoculum. We correct for this "background" by
>
> $$m_g = m - m_b, \quad (2)$$
>
> where $m_b$ is the average number of mutants per compartment in the inoculum (as determined by direct plating), and $m_g$ is the corrected average number, i.e., the mutational events occurring during the growth in the compartments. This can be converted to the mutational events per cell per generation, $M$, by
>
> $$M = m_g/2C \quad (3)$$

where $C$ is the number of cells per compartment after growth has ceased in the limiting medium. The factor of two in the denominator is necessary because the number of cell generations in the history of a culture is approximately twice the final number of cells. Actually, the proper value for this numerical factor depends upon the point(s) in the cell cycle at which growth is terminated in the limiting medium and also upon the distribution of mutation production over the cell cycle. Since it enters only as a scale factor in all mutation rate calculations, relative mutation rates are unaffected by the value used.

This method for determining mutation rates is due to Luria and Delbrück (1943). The principal advantage of the method is that the results are not affected by many types of selection. Since we only score the presence or absence of a mutational event in a culture, it is clearly irrelevant whether the mutants grow faster or slower than non-mutants.

In those experiments where the mutants are further analyzed into categories the mutation rate may be partitioned by

$$M_i = f_i M \qquad (4)$$

where $M_i$ is the mutation rate (per cell per generation) for the $i$th category and $f_i$ is the fractions of the mutants tested which were found to be in the $i$th category.

A sample calculation is contained in Appendix 1.

DNA Isolation.

DNA for PCR analysis and other small-scale preparative work was isolated from yeast by the method of Polaina and Adam (1991). Overnight cultures were grown in YEPD at 30° C. A 1.5-ml microfuge tube was filled with culture and centrifuged to pellet the cells. The cells were resuspended in 100 µl of TE-SDS buffer (10 mM Tris, 1 mM EDTA; pH 7.5 plus 3% SDS) and incubated on the benchtop for 15-30 minutes with occasional mixing. The cells were diluted with 500 µl of TE, then 600 µl of phenol-

chloroform-isoamyl alcohol (50:49:1) was added. The mixture was vortexed for 1 minute, then centrifuged for 10 minutes at room temperature. A 500 µl aliquot of the aqueous phase was transferred to a new microfuge tube, and DNA was precipitated with 10 µl 5 M NaCl, and 500 µl isopropanol. The DNA was centrifuged, washed with 70% ethanol, and dried on the benchtop until the pellet became translucent. The nucleic acid pellet was resuspended in 50 µl TE. One µl was used for subsequent PCR analysis.

Bacterial plasmid DNA was isolated using the alkaline lysis method (Sambrook *et al.*, 1989).

Construction of carbon regulation mutants in Saccharomyces cerevisiae.

The *G418*$^R$ alleles of *snf1*, *hxk2*, and *mig1* were constructed in *Saccharomyces* by replacing the endogenous coding region of each gene with the *Kan*$^R$ coding region derived from the vector pET9c (Studier *et al.*, 1990). This results in the expression of aminoglycoside phosphotransferase-3' (I), which has been shown previously to be a useful selectable marker in yeast (Webster and Dickson, 1983), conferring resistance to G418 or Geneticin.

*Saccharomyces* can catalyze recombination events between DNA molecules with very short (<10 base pairs) regions of homology (Ahn *et al.*, 1988). This ability allows integration of foreign DNA provided the integrating fragment has a small region of homology (30-45 base pairs) on the ends of the incoming fragment (Baudin *et al.*, 1993; Manivasakam *et al.*, 1995). The replacement cassettes were constructed by synthesizing two primers for each locus which contained 45 base pairs of sequence from the target locus and 20 base pairs of sequence from the *Kan*$^R$ gene in pET9c (Table 4). The primers

were analyzed and optimized using the Web-Primer PCR analysis service located at the Saccharomyces genome database (http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer). The primers used in this thesis are shown in Table 4.

PCR reactions were carried out using matching primers with the pET9c vector as template to generate a product which contains the entire coding sequence of the $Kan^R$ locus, with 45 base pair extensions which are homologous to the *Saccharomyces cerevisiae* target locus. The insert was amplified with Taq DNA polymerase (Promega) according to manufacturer's suggested conditions: 200 nmol of each dNTP, 2.5 mM $MgCl_2$, 1X buffer, 10 ng/ml of each primer, 2.5 units Taq polymerase plus 10 ng plasmid pET9c as template. The amplification was performed in a Stratagene Robocycler, under the following reaction conditions: 94° C, 4 min; (99° C, 30 sec, 55° C, 30 sec; 72° C, 2 min) x 30; 72° C, 4 min. This PCR product was purified by gel electrophoresis and elution with Glassmilk (Bio101).

Transformation of yeast was performed according to the method of Gietz and Schiestl (1995). Cells are grown in liquid YEPD to late log phase (1-2 $x10^7$ cells per ml). The cells are centrifuged, and washed with sterile water, then resuspended in 1.0 ml 100 mM lithium acetate, transferred to a microfuge tube and centrifuged again. This pellet is resuspended in 100 mM lithium acetate to a concentration of 2 x $10^9$ cells/ml. 50 µl of cell suspension is overlaid with 240 µl of 50% PEG 3350, 36 µl of 1.0 M lithium acetate, 25 µl of single stranded DNA, and 50 µl containing 1 µg of transforming DNA in water. The mixture is vortexed at high speed until the cells are evenly dispersed. The transformation tubes are incubated at 30° C for 30 minutes, then transferred to 42° C for 15 minutes. The cells are pelleted, and resuspended in 1 ml of sterile water.

Transformants are selected by plating on 200 mg/l G418. $G418^R$ colonies were restreaked on YEPD plates and then replica-plated to selective media. Strains that carry *snf1* are unable to grow on non-glucose media. Strains carrying the *hxk2* or the *mig1* mutations are able to grow on 2-deoxyglucose/glycerol plates. The 2-deoxy-glucose can be taken up by the cell and phosphorylated once, triggering glucose repression, but cannot be used as a carbon source (Zimmerman and Scheel, 1977). Wild-type cells die under these conditions. In the mutants, catabolite repression is not functional, allowing the cells to use the glycerol as a carbon source. All $G418^R$ transformants tested also exhibited the expected phenotype. The insertion of the $Kan^R$ marker into the target genes and replacement of the native gene was also confirmed by PCR using primers which flanked the region replaced by the construct (Table 4).

UV mutagenesis and survival tests.

Cells were grown overnight in rich media to saturation. The culture was counted by haemocytometer, washed with sterile water, and resuspended in water to $2 \times 10^6$ cells/ml. 40 mls of this suspension was placed into a deep petri dish, set upon a magnetic stirrer, and stirred slowly during the entire exposure period. The UV source was a 30-watt GE30T8 germicidal bulb (General Electric) set approximately 40 cm above the plate. The UV fluence was measured as 9 ergs/sec using a Latarjet UV dosimeter (Latarjet *et al.*, 1953).

The cell suspension was irradiated in 30 second bursts. Between irradiations, 2.0 mls was withdrawn and diluted in 6 mls sterile water to $5 \times 10^5$ cell/ml. This suspension was plated onto defined media lacking tryptophan (0.5 ml per plate) to assess induced

reversion frequency. The diluted suspension was further diluted to 500 cells per ml, and plated on YEPD(K) to track survival.

Survival plates were scored at 4-5 days, and reversion plates were scored at 7-8 days post-irradiation. Reversion frequencies are expressed as revertants per $10^6$ survivors and plotted versus UV exposure. Data shown is the average of two or three separate experiments.

DNA sequencing of the *trp1-11* allele in *Kluyveromyces lactis*.

Total cellular DNA was isolated from JA6 as described above. The *trp1-11* locus was amplified from this DNA using primers KT1-3' and KT1-5' derived from the known sequence of the *TRP1* locus (Stark and Milner, 1989. Primers were designed and evaluated using the *Amplify* program for the Macintosh. The product of the PCR reaction was gel-purified using Geneclean (Bio101). The purified DNA was sequenced using Sequenase 2.0 (Amersham) with $^{33}$P-ddNTP as per manufacturer's instructions. The reactions were electrophoresed into a 6% polyacryamide-urea gel using a sequencing gel apparatus (Tyler). The gel was transferred to filter paper, dried, and exposed to Kodak X-Omat film. Sequences were read from the film manually. Four primers were used to generate the sequence (Table 4), which was assembled into a single sequence using SeqVu for the Macintosh. The *trp1-11* sequence was aligned with the *TRP1* sequence using SeqVu to identify sequence differences. The completed *trp1-11* sequence, aligned with the Genbank sequence (Accession #X14230; Stark and Milner, 1989), is shown in Appendix 2.

Table 1. Strain genotypes of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* used in this study.

| Strain | Genotype | Source |
|---|---|---|
| ***Saccharomyces cerevisiae*** | | |
| LL9-3B | *MATa ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 ura3-13* | Stock |
| XV185-14C | *MATa ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48* | Stock |
| XV185-6A | *MATα ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48* | Stock |
| MIKY45[a] | *MATa ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 mig1::Kan$^R$* | This study |
| MIKY46[a] | *MATa ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 snf1:: Kan$^R$* | This study |
| MIKY47[a] | *MATa ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 hxk2:: Kan$^R$* | This study |
| HP152-8A | *MATa ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 rad51* | Stock |
| YO418-5D | *MATα ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 rad3-12* | Stock |
| XV363-1A | *MATa ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 rad1* | Stock |
| YO151-2D | *MATa ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 rad6-1* | Stock |
| HP190-2B | *MATa ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 rad18-1* | Stock |
| XV2023-11A[c] | *MATa ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 csm1-1* | Stock |
| XV2023-8C[c] | *MATα ade2-1 arg4-17 his1-7 hom3-10 lys1-1 trp5-48 rev3-Δxx* | Stock |
| ***Kluyveromyces lactis*** [b] | | |
| JA6 | *MATa ade1 ade2 trp1-11 uraA* | K. Breunig |
| JA6-*fog1*Δ | *MATa ade1 ade2 trp1-11 uraA fog1::URA3* | I. Ferrero |
| JA6/207 | *MATa ade1 ade2 trp1-11 uraA fog2* | I. Ferrero |
| JA6/151 | *MATa ade1 ade2 trp1-11 uraA rag5-151* | I. Ferrero |
| JA6/239 | *MATa ade1 ade2 trp1-11 uraA rag5-239* | I. Ferrero |
| yIG2 | *MATa ade1 ade2 trp1-11 uraA mig1::URA3* | I. Georis |
| MIKY49 | *MATa ade1 ade2 trp1-11 uraA fog1::URA3 sfg1-1* | This study |

[a] These strains are isogenic to XV185-14C except as indicated.
[b] All the mutant *K. lactis* strains are derived from JA6.
[c] These two strains derive from the same diploid parent strain.

Table 2. Amino acid and base supplementation for defined media.

| Amino acid or base | Stock solution (g/L)[a] | Volume added per litre (mls) | Final concentration (mg/L) |
|---|---|---|---|
| Adenine | 1 | 20 | 20 |
| L-Arginine | 2 | 10 | 20 |
| L-Histidine | 2 | 10 | 20 |
| L-Isoleucine | 3 | 10 | 30 |
| L-Leucine | 5 | 10 | 50 |
| L-Lysine | 3 | 10 | 30 |
| L-Methionine | 2 | 10 | 20 |
| L-Phenylalanine | 5 | 10 | 50 |
| L-Threonine | 5 | 10 | 50 |
| L-Tryptophan[b] | 2 | 10 | 20 |
| L-Tyrosine | 0.75 | 40 | 30 |
| Uracil | 2 | 10 | 20 |
| L-Valine | 15 | 10 | 150 |

[a] All solutions are filter-sterilized.
[b] Tryptophan is light-sensitive.

Table 3. Limiting concentrations for compartmentalization tests.

| Marker selected | Limiting amino acid(s) | Final concentration (mg/l) |
|---|---|---|
| ***S. cerevisiae*** | | |
| *his1-7* | histidine | 0.4 |
| *arg4-17* | arginine | 1.0 |
| | adenine | 5.0 |
| *ura3-13* | uracil | 0.35 |
| ***K. lactis*** | | |
| *trp1-11* | tryptophan | 0.4 |

Table 4. PCR Primers used during this study.

| Primer | Sequence (5'→3') |
|---|---|
| HXK2-F | CCGTGATGTCTCTTTGTTGCAC |
| HXK2-R | CGTCACGAATAAATCCCGTGAA |
| MIG1-F | TGGCTTCTCAGGAAACCTGT |
| MIG1-R | GGAAGGGCAACGGTAAACTT |
| SNF1-F | GTGTCCAAACAGTCATTCAGGAAG |
| SNF1-R | TTTTTCTCATAGAGCGTGAAATTTG |
| HXK2-KF [a] | AGGAATATAATTCTCCACACATAATAAGTACGCTAATTAAAT AAAgttatgagccatattcaacg |
| HXK2-KR [a] | AAAACATGTTCACATAAGTAGAAAAAGGGCACCTTCTTGTTG TTCactaccgcattaaagcttatc |
| MIG1-KF [a] | ACTACACGAGAGTTGAGTATAGTGGAGACGACATACTACCA TAGCgttatgagccatattcaacg |
| MIG1-KR [a] | TGTCTATTGTCTTTTGATTTATCTGCACCGCCAAAAACTTGTC AGactaccgcattaaagcttatc |
| SNF1-KF [a] | TTTTTTTTGTAACAAGTTTTGCTACACTCCCTTAATAAAGTCA ACgttatgagccatattcaacg |
| SNF1-KR [a] | AGATGTTGCAAATACGTTACGATACATAAAAAAAAGGGAAC TTCCactaccgcattaaagcttatc |
| KT1-A | CTTTGATCCCATCTTCAATTCC |
| KT1-B | GGAATTGAAGATGGGATCAAAG |
| KT1-5' | AAAAATTGAAGTATCAAACATCAG |
| KT1-3' | TATGTGTAATTGAAGAAAGATACG |

[a] Yeast sequence is in UPPER CASE, plasmid sequence is in lower case.

## Results

Spontaneous mutation rate is investigated by a number of methods most of which involve the growth of several independent cultures and the quantification of the proportion of the culture that has mutated at a given locus. Statistical analysis of the raw data yields a mutation rate expressed as mutations per cell per generation. The most utilized method of analysis is the fluctuation test, first proposed and tested by Luria and Delbruck (1943). This test requires a large number of cultures (>35) in order to be a valid measurement of mutation rate. This requirement makes the technique unwieldy for large-scale screening of conditions or mutants that affect mutation rates. In this study, I have employed two techniques: the Lassie test and the compartmentalization test. Both tests have been described in detail and compared with other forms of mutation rate analysis previously (von Borstel *et al.*, 1971; von Borstel, 1978).

The Lassie test is a qualitative test that is utilized for the rapid evaluation of relative mutation rates. The test comprises two plates of synthetic media, one (the dropout plate) lacking a specific metabolite needed by the cells to be plated (commonly an amino acid), and another plate (the limiting plate) containing a small amount of the required metabolite. The two plates are spread with an equal number of cells, and incubated until colonies appear. The dropout plate will generate colonies from pre-existing revertants, and the limiting plate will generate revertant colonies which arose prior to plating and during the growth that takes place until the small amount of supplied metabolite is exhausted. The difference in colony number between the dropout plate and the limiting plate is the Lassie score. Changes in mutation rate are identified by

corresponding changes in the Lassie score. The test can be quantitated reasonably well by estimating the number of cells per plate following growth.

The compartmentalization test was developed by von Borstel *et al.* (1971) as an improved method for the study of mutation rates since it eliminates residual growth as a complicating factor. The test is an extension of the fluctuation analysis described by Luria and Delbruck (1943). A large number of independent cultures arrayed in 6x4 tissue culture boxes, typically 500, are seeded with a small number of cells. The cultures contain a limiting amount of one metabolite that allows several generations of growth but not complete exhaustion of the media. The majority of the cells in the culture will arrest at the point at which the limiting metabolite is exhausted. Cells that have mutated during the growth phase such that they can grow without supplementation continue to grow, forming colonies above the background lawn of cells. The limiting metabolite is set in such a way that a large number of the cultures do not generate colonies. This limitation allows the application of the Poisson distribution to calculation of the mutation rate. (See Material and Methods, and Appendix 1)

In practice, the compartmentalization test is extremely precise, allowing detection of variation as small as 5%. The historical reliability of the test is demonstrated in Table 5 (taken from von Borstel *et al*, 1998, and unpublished data). The experiments shown for the *his1-7* locus were performed during an 8-month period. The mean of the seven experiments is $6.34 \times 10^{-8}$ reversions/cell/generation, the standard deviation is 0.297, and the coefficient of variation is 4.7%. The coefficient of variation (CV) is used as a measure of the precision of the test. Smaller numbers are indicative of greater precision.

By comparison, a CV of 20% has been shown to be the level of scatter obtainable under optimal conditions using the fluctuation test (Kendal and Frost, 1988).

Validation of experimental protocol

The compartmentalization test was originally developed for use with *Saccharomyces cerevisiae* strains grown on glucose. *Saccharomyces cerevisiae* prefers to ferment glucose, and normally uses very little oxygen during growth on glucose. This permitted the use of sealed, non-shaking, liquid cultures in prior applications of the test. The use of carbon sources that require aerobic respiration required modification of the test to increase aeration of the cultures. This was accomplished by the addition of 0.7% agar to keep the cells at the surface of the culture media, and increased circulation of air around unsealed boxes. Evaporation of the media was limited by incubating the cultures at 90% humidity. To confirm that the test is still reliable under these conditions, I compared the results of separate tests of spontaneous reversion at the *his1-7* and the *arg4-17* loci in S. cerevisiae using the coefficient of variation (coefficient of variation (CV) = mean/standard deviation) as a measure of scatter in the data.

The rates for each test are shown in Table 6 and the calculated CV for each carbon source and locus. The amount of scatter in the experiments shown in Table 6 is larger than that observed in the submerged cultures previously (see above) ranging from 1.93 – 62.9%, with a median CV of 17.3%. This CV compares well with the minimum value of 20% obtainable with the fluctuation test (see above). The high variation in the

*arg4-17* (suppressor)-glycerol studies is due to the low numbers of revertants recovered, which forces large changes in the calculated rate when small changes in revertant frequency are observed.

The increase in variation relative to the earlier work (von Borstel *et al.*, 1998) may be due to the more variable culture conditions. Oxygenation did not appear to be equivalent in all compartments, and exterior compartments were subject to drying stresses during the experiment. These problems were recognized early on and culture conditions were adjusted to the greatest extent possible by increasing the relative humidity and air circulation, and testing several culture boxes, but complete uniformity could not be attained. Experiments were scored in a manner that minimized possible effects of compartment specific variation in growth and mutability.

On the basis of the CV results, differences of 2-fold in spontaneous mutation rate are accepted as being real differences throughout this work. The potential variation present in the compartmentalization test is limited primarily by the relative size of the $P_0$ and $P_1$ classes of the distribution. When the two classes are similar, then changes in the absolute size have less effect. When the two classes ar not equivalent, then small changes can have a very large effect as is observed in the *arg4-17* (suppressor) glycerol results (Table 6). The observed CV in this case is 62.9%. In order to prevent the false assumption of a difference between two results when the size of the $P_0$ class is limiting the precision of the test, we will assume that 100% changes are indicative of a true difference in spontaneous mutation rate.

Estimation of spontaneous mutation rate in *S. cerevisiae*

Lassie tests The Lassie test was used for the initial evaluation of the effect that carbon source has upon the spontaneous mutation rate. A series of Lassie plates were made that differed only in the carbon source that was present. The Lassie score for each carbon source was determined using *S. cerevisiae* strain XV185-14C, testing the reversion of *his1-7*. The results are shown in Table 7.

There is no real variation in the background $HIS^+$ frequency between carbon sources as indicated by the colony numbers in the first column. The number of colonies that arise on the limiting plates is highly variable, showing a 47-fold difference between the highest and lowest Lassie scores (glucose versus acetate).

Each of the carbon sources was also tested in combination with glycerol. This test was included because of results discovered as part of a separate project (Hamilton and von Borstel, 1996), where there was evidence that the presence of glycerol in arrested cultures was able to both increase survival at 26° C and appeared to reduce mutation frequencies. Now I have found that the presence of glycerol affects the observed Lassie score in a carbon-source-specific manner. There does not appear to be any effect on Lassie in cultures grown on glucose, ethanol, or acetate in combination with glycerol. A slight reduction in mutation rate is observed when glycerol is combined with fructose, and a 50% drop in Lassie score when glycerol is added to galactose grown cells. The addition of glycerol to maltose cultures caused an increase in the Lassie score.

Compartmentalization tests. The results of compartmentalization tests with XV185-14C are shown in Table 8. The general trend observed in the Lassie tests is maintained. The spontaneous mutation rate is highest in glucose-grown cultures and lowest for ethanol. The carbon sources appear to cluster naturally into three groups with similar mutation rates. The highest mutation rates are observed in cultures grown in glucose, fructose, or sucrose. A second group has mutation rates approximately 3-fold less than the first group. This cluster includes glycerol, galactose, and xylose. The third group exhibits a further 3-fold reduction in mutation rate, and includes ethanol, acetate and maltose.

Different alleles The *his1-7* allele is a missense allele which reverts at a variety of different sites within the *his1* locus, and by all possible base substitutions (von Borstel *et al.*, 1998). The broad spectrum of possible reversion sites for *his1-7* would suggest that the effect that I have observed would be easily generalized to other allele types. This expectation was borne out by testing three other loci, which carry known mutations that are different than the *his1-7* mutation (Table 9).

The *ura3-13* allele is a spontaneous mutation of the *URA3* locus. It is a direct duplication of 23 base pairs which reverts easily (von Borstel *et al.*, 1993). It has previously been shown to exhibit adaptive characteristics (Liviero and von Borstel, 1994). Table 9 shows the effect of changes in carbon source on reversion of this allele. The effects observed for this allele are more extreme than the effects observed for the *his1-7* allele. The mutation rate for *ura3-13* in glycerol-grown cultures is far lower, approximately a 37-fold decrease. It should be noted that these experiments were performed in submerged liquid cultures instead of solid medium.

The *arg4-17* allele is a Type II suppressible ochre mutant which is easily suppressed by a variety of tRNA anticodon mutations. Locus revertants can be distinguished from tRNA suppressors by co-suppression of the *ade2-1* allele that is also present in XV185-14C. The *ade2-1* mutant confers a red colony color due to the accumulation of a pigmented metabolic intermediate. Suppressor tRNAs revert both the *arg4-17* and the *ade2-1* resulting in the outgrowth of white $ARG^+$ colonies. Locus revertants appear as red colonies.

The reversion rate of the *arg4-17* allele is reduced by growth on non-glucose carbon sources in the same manner as *his1-7* reversion. The spontaneous reversion rate in glucose-grown cultures is much greater than in glycerol-grown cultures (Tables 6, 9). The majority of the recovered revertants are suppressor mutants (84%, Table 9). The change in carbon source affects the suppressor frequency much more so than the locus revertant frequency, causing a 12-fold reduction in the locus reversion frequency, but a 52-fold reduction in suppressor frequency.

Estimation of spontaneous mutation rates in *Kluyveromyces lactis*

*Kluyveromyces lactis* was used for the investigation of the carbon regulatory effect. The carbon regulation system in this species has been shown to be very similar in structure to the *Saccharomyces cerevisiae* system (Gancedo, 1998), but there is no genetic redundancy for the components of the regulatory cascade. We used the *Kluyveromyces lactis* strain JA6 for our experiments since it is known to be glucose-

repressible (Breunig, 1989), and many carbon metabolism mutants have been isolated in the JA6 background (Goffrini *et al.*, 1989; Wesolowski-Louvel *et al.*, 1992; Goffrini *et al.*, 1996).

Compartmentalization tests. Strain JA6 was tested for the rate of spontaneous reversion of the *trp1-11* allele when grown on different carbon sources. This allele was sequenced as part of this work, and comparison to the published sequence showed it to be a CG→TA transition that creates an ochre nonsense codon at the 51$^{st}$ codon (Appendix 2).

The results are shown in Table 10. In contrast to the *Saccharomyces* results, the spontaneous reversion rate on glucose is the lowest rate observed. The highest rates are observed in ethanol and acetate cultures. There is a sharp separation between the rates observed on most carbon sources and the rates observed on ethanol and acetate. As was observed in the *Saccharomyces cerevisiae* results, carbon sources that are more commonly used as a food source such as lactose and sucrose generate spontaneous mutation rates that are more like the rate observed in glucose cultures. The differences in spontaneous mutation rate are not as extreme as they are in S. cerevisiae, howver, the same trend in changes is apparent, with ethanol and glucose observed as extremes in spontaneous mutation rate, with glycerol and xylose as intermediate values.

Spontaneous mutation rates in carbon regulation mutants.

The influence of the carbon source on spontaneous mutation rates observed above suggests that some aspect of carbon metabolism is affecting the spontaneous mutation

rate. The transition from glucose to another carbon source causes the release of glucose-mediated repression, a transcription regulation system which prevents the expression of genes which are not necessary for the catabolism of glucose. This regulation operates through a regulatory cascade (Fig 1).

If carbon metabolism has an effect upon spontaneous mutation, then strains which are deficient in carbon regulation may have an altered rate of mutation. The carbon regulatory system in Saccharomyces is redundant, with multiple transporters, kinases, and repressors (Gancedo, 1998; Boles, 1997; and references therein). This characterstic appears to be the result of a tetraploid phase during the evolution of the species (Wolfe and Shields, 1997). Genetic background can also have a strong effect on the penetrance of some defects in carbon regulation (Trumbly, 1992). These observations indicated that isogenic strains were necessary for investigation of the effects of carbon regulation on spontaneous mutation.

Three genes were selected for study; *HXK2*, *SNF1*, and *MIG1*. These genes have clear phenotypes, and have been shown to be central to the regulatory cascade. Cells lacking *HXK2* or *MIG1* are unable to establish glucose repression (Neigeborn and Carlson, 1987; Schüller and Entian, 1991). Strains lacking *SNF1* in both *S. cerevisiae* and *K. lactis* are unable to induce genes which are being repressed, even in the absence of glucose (Carlson, 1987). Isogenic mutants were constructed by replacing the coding sequence for each gene with the coding region of the kanamycin resistance gene from pET9c (see Materials and Methods). This permits expression of the resistance gene, which makes the cell resistant to G418, allowing direct selection of cells carrying the gene replacements.

The results of compartmentalization tests with the three *S. cerevisiae* deletion strains are shown in Table 11. The *snf1* mutant has no effect upon mutation rates compared to the isogenic parent strain, XV185-14C. The *hxk2* and *mig1* strains, which have similar effects upon carbon regulation, have similar effects upon spontaneous mutation. There is no significant reduction in mutation rate on glucose and sucrose relative to the wildtype (Table 8), a minor increase in mutation rate on galactose, and strong negative effects on mutation rate in these strains when grown on glycerol, maltose and ethanol. The *mig1* mutant has a much more suppressive effect on spontaneous mutation in maltose and ethanol cultures.

The interaction between glycerol and ethanol is different for these two strains. In the *mig1* strain, the mutation rate in the mixed glycerol/ethanol culture is similar to the glycerol culture, as it is in the wildtype strain (Table 11). In contrast, in the *hxk2* strain growth on ethanol and glycerol together results in a lower mutation rate than either carbon source alone.

<u>*Kluyveromyces lactis*</u> The carbon regulation system mediates the transition between two states: repression on (glucose present) and repression off (no glucose). Mutants in the pathway have either a non-inducible phenotype, unable to grow on non-glucose carbon sources; or a non-repressible phenotype, able to use alternate carbon sources in the presence of glucose.

Non-repressible carbon regulation mutants in *Kluyveromyces lactis* are represented here by *mig1* and *rag5*. The *rag5* gene encodes the hexokinase that is the first regulatory step of the signal cascade (Prior *et al.*, 1993). The *mig1* gene encodes the

primary repressor protein which blocks transcription of the genes required for catabolism of non-glucose carbon sources (Cassart *et al.*, 1993). Both mutations permit growth under conditions where glucose is present but cannot be utilized by the cell.

Strains carrying the *fog1* or *fog2* mutations are unable to grow on several carbon sources due to defects in the induction of carbon-source-specific genes. The *FOG2* gene is homologous to and functionally complements the *snf1* mutant of *S. cerevisiae*, which is known to be necessary for full expression of several glucose-repressed genes (Goffrini *et al.*, 1996). The *FOG1* gene shares homology with several *S. cerevisiae* genes which are known to interact with *SNF1*, such as *GAL83 (=SPM1), SIP1,* and *SIP2 (=SPM2).*

The *fog* strains have been previously reported to be unable to grow on glycerol (Goffrini *et al.*, 1996); I found that this phenotype is due in part to a nutritional deficit which can be rescued by additional yeast nitrogen base in defined media, or additional yeast extract in rich media. Accordingly, all experiments reported here contain double the manufacturer's recommended amounts of yeast nitrogen base.

The *mig1* and *rag5* mutants were grown on glucose, and then plated on several carbon sources to test for effects on spontaneous mutation rates. The increased rate of spontaneous mutation observed in the wildtype in most non-glucose carbon sources suggested that the release of repression might contribute to the effect. I therefore expected that the removal of repression would cause an increase in mutation rate in glucose-grown cultures.

This expectation was not observed (Table 12). The spontaneous mutation rate was not changed on glucose or galactose, and declined on other carbon sources relative to

the rate observed in the wildtype strain. The greatest decline occurred in glycerol cultures for both *rag5* and *mig1* cultures.

The *fog1* and *fog2* mutants grow well on glucose, and slowly on glycerol. They are unable to grow on any other carbon source. These mutants were not expected to have an effect upon spontaneous mutation rate in glucose. There might be a possible antimutagenic effect in glycerol-grown cultures if the increase in mutation rate were due to a stimulatory effect of the catabolite derepression event.

Both strains show a strong mutator effect when plated on glucose (Table 13). The *fog1* strain shows a 14-fold increase in mutation rate, and the *fog2* strain shows a 6-fold increase. Neither strain shows any change in mutation rate when grown on glycerol. A spontaneously arising suppressor of the *Δfog1* allele (*SFG*; suppressor of ***fog***) was isolated that permitted growth of the *fog1* strain on non-glucose carbon sources. The *fog1 SFG* strain had a reduced mutator phenotype on glucose, but an antimutator phenotype on glycerol.

Interactions between DNA repair and carbon metabolism

The observations above strongly support the hypothesis that changes in carbon metabolism cause changes in DNA metabolism. The source of the change could be due to direct changes in DNA repair capacity, or to variation in the rate of DNA damage, possibly caused by endogenous metabolites. Direct identification of endogenous

mutagenic metabolites or reliable detection of spontaneous lesions is not yet possible, so I decided to test the effects that carbon metabolism has upon DNA repair.

If we hypothesize that the carbon-mediated change in mutation rate is due to a change in the repair capacity of the cell, then cells grown on different carbon sources may exhibit altered sensitivity to DNA damaging agents. This could manifest itself as an increased sensitivity to exogenous DNA damage, or as a reduction in exogenously-induced reversion frequencies..

UV irradiation and carbon source. The wildtype *Kluyveromyces lactis* strain JA6 was pregrown in rich media containing different carbon sources. The cultures were irradiated in water, then plated on media containing the same carbon source as before irradiation. The results are shown in Figure 2. Survival on glucose and glycerol is similar, but ethanol-grown cells are more sensitive to the killing effect of UV light. This effect is not due to the post-irradiation medium, but to the pre-irradiation carbon source (Figure 3). Cultures grown in ethanol show the same survival kinetics, despite recovering on different carbon sources.

The reversion of the *trp1-11* allele by UV irradiation was also tested in a similar manner. The induction of $TRP^+$ colonies by UV light is affected by the carbon source available (Figure 4). The glucose and glycerol kinetics of $TRP^+$ induction do not differ, but the ethanol culture appears to saturate at about 120 seconds of UV exposure. Interestingly, this effect does depend upon post-irradiation media. Plating an ethanol-grown culture on glucose following irradiation suppresses the saturation effect. Glucose grown cultures plated on ethanol following irradiation show a reduction in $TRP^+$

frequency, but not the upper limit to *TRP*$^+$ recovery observed in the ethanol to ethanol experiments (Figure 5).

UV irradiation of catabolite regulation mutants. The effect of catabolite regulation on UV survival was tested using the *mig1* and *fog1* mutants of *K. lactis*. Survival of the *fog1* mutant is not different from the wildtype grown on glucose (Figure 6). Induction of *TRP*$^+$ reversion is indistinguishable from wildtype kinetics as well (Figure 7).

The *mig1* strain has survival kinetics different than wildtype. It suppresses the increased sensitivity to UV killing observed in the ethanol-grown strains (Figure 8), and increases the survival of glycerol- and glucose-grown cells as well. Reversion of *trp1-11* is increased at all UV doses in the *mig1* mutant grown on glycerol but not for glucose-grwon cells (Figure 9). An increased frequency of *trp1-11* reversion is observed for *mig1* cells grown on ethanol at low doses, but the saturation effect observed for ethanol grown wildtype cultures is still present in the *mig1* strain. It occurs at an earlier dose (60 sec vs 120 sec in the wildtype), but at the same approximate frequency of *TRP*$^+$ colonies/survivor.

Spontaneous mutation rates in DNA repair-deficient strains of *S. cerevisiae*.

If growth on a non-glucose carbon source causes a specific change in one DNA repair pathway, then we would expect that strains carrying an additional DNA repair defect in a separate pathway would behave as though both pathways are absent, producing a phenocopy of the double mutant. Almost all DNA repair mutants exhibit an associated increase in spontaneous mutation rates. The decline in spontaneous mutation

rate observed in *S. cervisiae* suggests that DNA repair is becoming more efficient. If this effect is due to increased use of a more reliable DNA repair system, then we expect that the lack of that DNA repair system would generate an increased mutation rate. The cultures grown on non-glucose carbon sources would have spontaneous mutation rates comparable to glucose cultures.

The mutation rate in DNA repair deficient strains of *S. cerevisiae* grown on different carbon sources is shown in Table 14. Strains representative of the three major DNA repair pathways are shown. Overall, the general trend seen in the wildtype is observed in the repair deficient strains as well. Glucose-grown cultures have the highest mutation rate, and ethanol-grown cultures the lowest.

A deficiency in excision repair (strain YO418-5D) leads to a mild increase in spontaneous mutation rate (Brychcy and von Borstel, 1977), and this effect is observed for glucose and ethanol (Table 14). However, glycerol-grown cultures of this strain have a very low mutation rate, below our detection level in these experiments. The cells present in the compartments can grow upon transfer to rich medium.

A deficiency in recombination repair (Strain HP152-8A) leads to a strong mutator phenotype that is observed in glucose or glycerol-grown cultures (Table 14). In ethanol-grown cultures, the mutation rate is below our detection level for these experiments. The cells in these compartments are still viable at 12 days post-plating as determined by resuspension and plating on rich media.

The third repair pathway investigated contains several genes which have been shown to be involved in misrepair events leading to mutation and is thus best described as mutagenic repair (Haynes and Kunz, 1981). Others (Prakash and Prakash, 1980) have

called this pathway post-replicative repair. The *rad18* mutant is a known mutator (von Borstel, 1971) and this effect is observed on all three carbon sources. The *rad6* mutant shows a reduction in spontaneous mutation rate on glycerol, much like the excision repair mutant. This strain is unable to grow on ethanol.

Some of the strains tested did show the phenotype predicted above when grown on different carbon sources. These strains are shown in Table 15. XV2023-11A is a putative wildtype strain. It shows a moderate mutator phenotype on glucose. The mutation rate is the same when grown on glycerol or ethanol. A similar phenotype is found in XV363-1A, which contains a *rad1* mutation and is defective in excision repair. When this strain is crossed to a strain which does exhibit the carbon source effect, XV184-6A, the resulting diploid exhibits the carbon source effect in Lassie tests. This diploid did not sporulate sufficiently well for further genetic analysis. The putative gene which gives rise to this phenotype has been tentatively assigned the name *csm1* (carbon regulation of spontaneous mutation).

XV2023-8C is derived from the same parent diploid strain as XV2023-11A but carries the *rev3* mutation, a polymerase required for DNA repair-induced mutagenesis. The spontaneous mutation rate in this strain is the same on all three carbon sources, but at a much lower rate than the other two strains, consistent with the previously described antimutator phenotype (Quah *et al.*, 1980). I do not know if this strain carries the *csm1* allele as well.

Figure 1. The glucose repression regulatory cascade in *Saccharomyces cerevisae*.

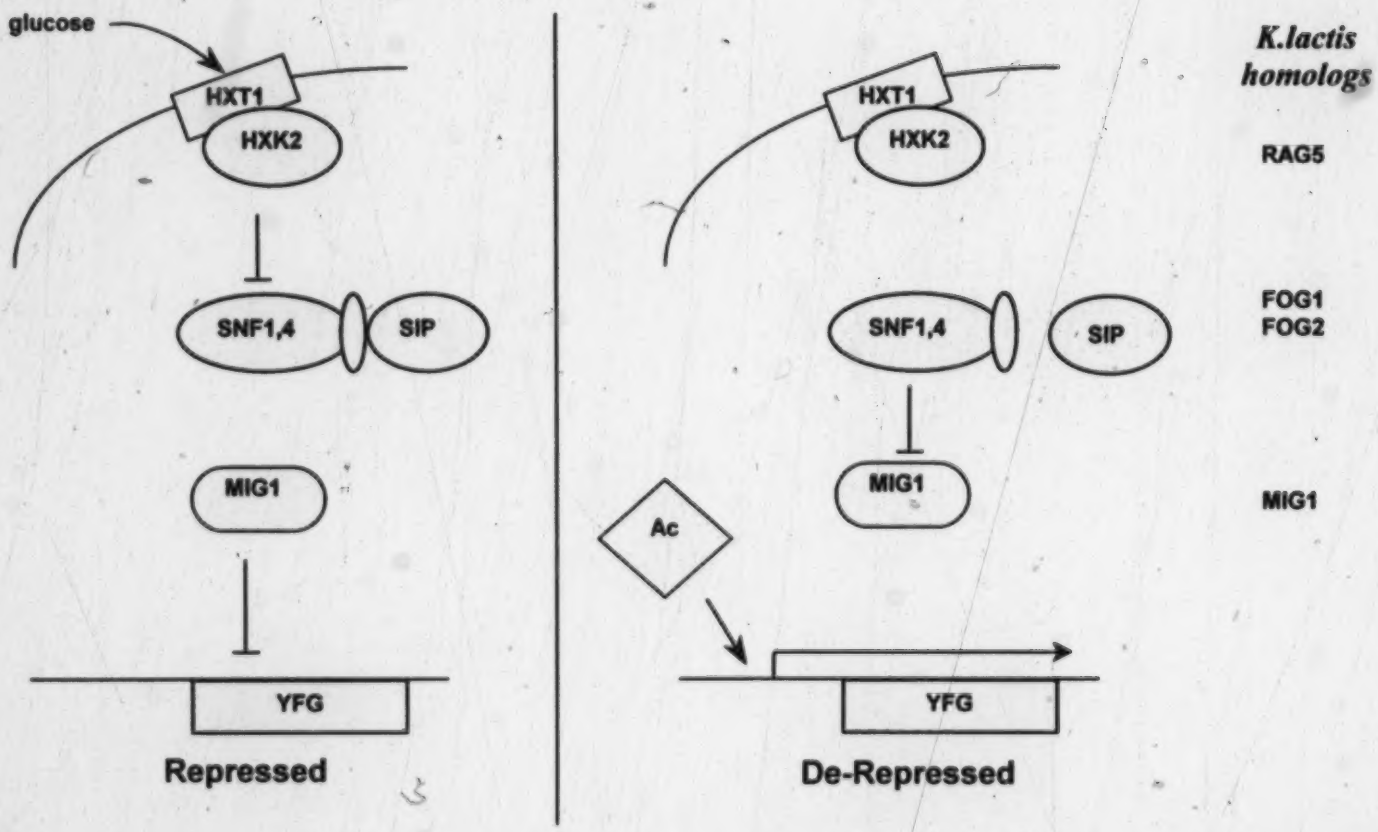


Table 5. Demonstration of repeatability of the spontaneous reversion rate assay: Results of repeated compartmentalization tests for base substitutions in *his1-7* of strain XV185-14C (taken from von Borstel *et al.*, 1998).

| Experiment number | Cells/ compartment ($\times 10^{-6}$) $C$ | Number of compartments $N$ | Number of compartments with mutants $N-N_0$ | Background mutants/ compartment $m_b$ | Mutation Rate ($\times 10^{-8}$) M [a] |
|---|---|---|---|---|---|
| 880921 | 1.91 | 498 | 105 | 0.032 | 6.16 |
| 881016 | 2.28 | 497 | 126 | 0.030 | 6.34 |
| 890401-1 | 2.99 | 482 | 146 | 0.031 | 6.01 |
| 890401-2 | 2.45 | 500 | 137 | 0.031 | 6.51 |
| 890401-3 | 2.80 | 500 | 148 | 0.020 | 6.24 |
| 890505 | 2.80 | 479 | 141 | 0 | 6.23 |
| 890514 | 2.31 | 488 | 134 | 0.021 | 6.92 |

[a] M = reversions/cell/generation.

Table 6. Estimation of the precision of the compartmentalization test as modified for this work.

| Carbon Source | Expt. # | Number of wells scored | Non-revertant wells | Cells per well ($\times 10^{-6}$) | Mutation rate[a] ($\times 10^{8}$) |
|---|---|---|---|---|---|
| *his1-7* | | | | | |
| glucose | 1 | 576 | 440 | 0.98 | 12.7 |
| | 2 | 528 | 386 | 0.86 | 17.6 |
| | 3 | 240 | 166 | 1.02 | 17.2 |
| | 4 | 639 | 401 | 1.78 | 13.1 |
| | | | Coefficient of variation[b] | | 17.2% |
| glycerol | | | | | |
| | 1 | 552 | 496 | 1.16 | 4.68 |
| | 2 | 566 | 527 | 0.99 | 3.40 |
| | 3 | 242 | 209 | 1.07 | 6.64 |
| | 4 | 591 | 539 | 0.95 | 4.85 |
| | | | Coefficient of variation | | 27.2% |
| *arg4-17* locus revertants | | | | | |
| glucose | 1 | 504 | 458 | 6.14 | 0.699 |
| | 2 | 528 | 427 | 5.80 | 0.685 |
| | 3 | 576 | 496 | 5.95 | 0.922 |
| | | | Coefficient of variation | | 17.3% |
| glycerol | | | | | |
| | 1 | 294 | 292 | 5.14 | 0.049 |
| | 2 | 581 | 577 | 5.20 | 0.053 |
| | 3 | 574 | 569 | 6.70 | 0.059 |
| | | | Coefficient of variation | | 9.37% |
| *arg4-17* suppressor revertants | | | | | |
| glucose | 1 | 504 | 322 | 6.14 | 3.56 |
| | 2 | 528 | 301 | 5.80 | 3.70 |
| | 3 | 576 | 358 | 5.95 | 3.64 |
| | | | Coefficient of variation | | 1.93% |
| glycerol | | | | | |
| | 1 | 294 | 293 | 5.14 | 0.014 |
| | 2 | 581 | 576 | 5.20 | 0.072 |
| | 3 | 574 | 568 | 6.70 | 0.059 |
| | | | Coefficient of variation | | 62.9% |

[a] Mutation rate = mutations/cell/generation

[b] Coefficient of variation = standard deviation/mean

Table 7. Non-glucose carbon sources reduce Lassie scores for *his1-7* reversion in *Saccharomyces cerevisiae* strain XV185-14C.

| Carbon source | Dropout colonies | Limiting colonies | Lassie score[a] |
|---|---|---|---|
| glucose | 2 | 49 | 47 |
| glucose/glycerol | 5 | 54 | 49 |
| fructose | 1 | 45 | 44 |
| fructose/glycerol | 3 | 38 | 35 |
| galactose | 2 | 25 | 23 |
| galactose/glycerol | 0 | 10 | 10 |
| glycerol | 0 | 18 | 18 |
| maltose | 3 | 9 | 6 |
| maltose/glycerol | 0 | 12 | 12 |
| ethanol | 0 | 4 | 4 |
| ethanol/glycerol | 1 | 5 | 4 |
| acetate | 0 | 1 | 1 |
| acetate/glycerol | 0 | 2 | 2 |

[a] Lassie score is the difference in colony number between the two sets of plates. Higher numbers are indicative of higher spontaneous mutation rates. Cells were grown overnight in YEPD, then diluted to $5 \times 10^6$ cells/ml. Aliquots of 0.5 ml were spread on each of 4 plates, two of each type. Plates were incubated for 5 days at 30° C.

Table 8. The spontaneous mutation rate in *S. cerevisiae* strain XV185-14C is reduced by growth on non-glucose carbon sources in the compartmentalization assay.

| Carbon source | Number of wells scored | Non-revertant wells | Cells per well ($x 10^6$) | Mutation rate[a] ($x10^8$) |
|---|---|---|---|---|
| sucrose | 599 | 403 | 1.44 | 13.8 |
| glucose | 639 | 401 | 1.78 | 13.1 |
| fructose | 403 | 249 | 2.08 | 11.6 |
| galactose | 599 | 512 | 1.59 | 4.93 |
| glycerol | 592 | 539 | 0.95 | 4.85 |
| glycerol/ ethanol | 576 | 505 | 1.65 | 3.56 |
| xylose | 600 | 551 | 1.38 | 3.09 |
| maltose | 592 | 551 | 2.20 | 1.68 |
| ethanol | 591 | 559 | 1.66 | 1.63 |

[a] Mutation rate = mutations/cell/generation

Table 9. Growth on glycerol reduces the spontaneous reversion rate of missense, nonsense, and duplication alleles in *Saccharomyces cerevisiae* relative to glucose-grown cultures.

| Allele/ Carbon Source | Number of wells scored | Non-revertant wells | Cells per well ($\times 10^{-6}$) | Mutation rate[a] ($\times 10^{-8}$) |
|---|---|---|---|---|
| *ura3-13*[b,c] | | | | |
| glucose | 298 | 7 | 3.85 | 48.7 |
| glycerol | 191 | 176 | 3.09 | 1.32 |
| *his1-7*[d] | | | | |
| glucose | 576 | 440 | 0.98 | 12.7 |
| glycerol | 552 | 496 | 1.16 | 4.68 |
| *arg4-17* locus revertants[e] | | | | |
| glucose | 528 | 427 | 5.80 | 0.685 |
| glycerol | 581 | 577 | 5.20 | 0.053 |
| *arg4-17* suppressor revertants[e] | | | | |
| glucose | 528 | 301 | 5.80 | 3.70 |
| glycerol | 581 | 576 | 5.20 | 0.072 |

[a] Mutation rate = mutations/cell/generation

[b] This experiment was done using the original liquid protocol.

[c] *ura3-13* is a 23 base pair duplication in strain LL9-3B.

[d] *his1-7* is missense mutation in strain XV185-14C that reverts at many sites within the *his1* locus.

[e] *arg4-17* is an ochre-suppressible nonsense mutant in XV185-14C that reverts at the locus, or at a number of suppressor tRNA genes. The suppressor revertants are differentiated by co-suppression of the *ade2-1* mutation which changes the colony color from red to white.

Table 10. Reversion of *trp1-11* in *Kluyveromyces lactis* strain JA6 increases when grown on non-glucose carbon sources in the compartmentalization test.

| Carbon Source | Number of wells scored | Non-revertant wells | Cells per well ($x 10^6$) | Mutation rate[a] ($x10^8$) |
|---|---|---|---|---|
| glucose | 522 | 73 | 44.6 | 2.2 |
| galactose | 519 | 72 | 30.7 | 3.21 |
| sucrose | 430 | 56 | 26.9 | 3.79 |
| lactose | 481 | 80 | 22.7 | 3.94 |
| mannitol | 479 | 8 | 48.8 | 4.19 |
| xylose | 539 | 156 | 10.1 | 6.11 |
| glycerol | 641 | 13 | 29.1 | 6.69 |
| maltose | 556 | 94 | 8.1 | 11.0 |
| ethanol | 541 | 173 | 0.96 | 58.8 |
| acetate | 430 | 157 | 0.73 | 68.7 |

[a] Mutation rate = mutations/cell/generation

Table 11. Non-repressing carbon regulation mutants augment the anti-mutagenic effect of non-glucose carbon sources in *Saccharomyces cerevisiae*.

| Carbon Source | Number of wells scored | Non-revertant wells | Cells per well ($x 10^6$) | Mutation rate[a] ($x10^8$) |
|---|---|---|---|---|
| *snf1*[b] | | | | |
| glucose | 164 | 83 | 3.44 | 9.89 |
| *hxk2*[b] | | | | |
| glucose | 286 | 137 | 5.02 | 7.33 |
| sucrose | 352 | 162 | 3.91 | 9.94 |
| galactose | 240 | 112 | 4.14 | 9.20 |
| glycerol | 271 | 235 | 4.58 | 1.56 |
| glycerol/EtOH | 232 | 217 | 5.47 | 0.611 |
| maltose | 275 | 261 | 5.52 | 0.473 |
| ethanol | 301 | 285 | 5.61 | 0.487 |
| *mig1*[b] | | | | |
| glucose | 198 | 113 | 3.38 | 8.3 |
| sucrose | 324 | 209 | 3.62 | 6.06 |
| galactose | 251 | 126 | 4.50 | 7.66 |
| glycerol | 232 | 203 | 4.68 | 1.43 |
| glycerol/EtOH | 234 | 209 | 5.77 | 0.98 |
| maltose | 224 | 218 | 6.73 | 0.202 |
| ethanol | 227 | 224 | 6.00 | 0.111 |

[a] Mutation rate = mutations/cell/generation
[b] These strains are were constructed in and are isogenic to XV185-14C.

Table 12. Non-repressing strains of *Kluyveromyces lactis* have lower mutation rates than wildtype on non-glucose carbon sources in the compartmentalization test.

| Carbon Source | Number of wells scored | Non-revertant wells | Cells per well ($x 10^6$) | Mutation rate[a] ($x10^8$) |
|---|---|---|---|---|
| *mig1*[b] | | | | |
| glucose | 418 | 113 | 34.5 | 1.89 |
| glycerol | 350 | 194 | 49.6 | 0.59 |
| galactose | 210 | 55 | 16.4 | 4.07 |
| maltose | 254 | 120 | 7.9 | 4.74 |
| ethanol | 349 | 229 | 2.0 | 10.2 |
| *rag5-151*[b,c] | | | | |
| glucose | 243 | 110 | 18.2 | 2.17 |
| glycerol | 261 | 150 | 20.9 | 1.3 |
| maltose | 277 | 193 | 3.45 | 5.23 |
| ethanol | 235 | 179 | 0.59 | 24.3 |
| *rag5-239*[b,c] | | | | |
| glucose | 331 | 186 | 4.72 | 6.1 |
| glycerol | 373 | 87 | 38.1 | 1.9 |

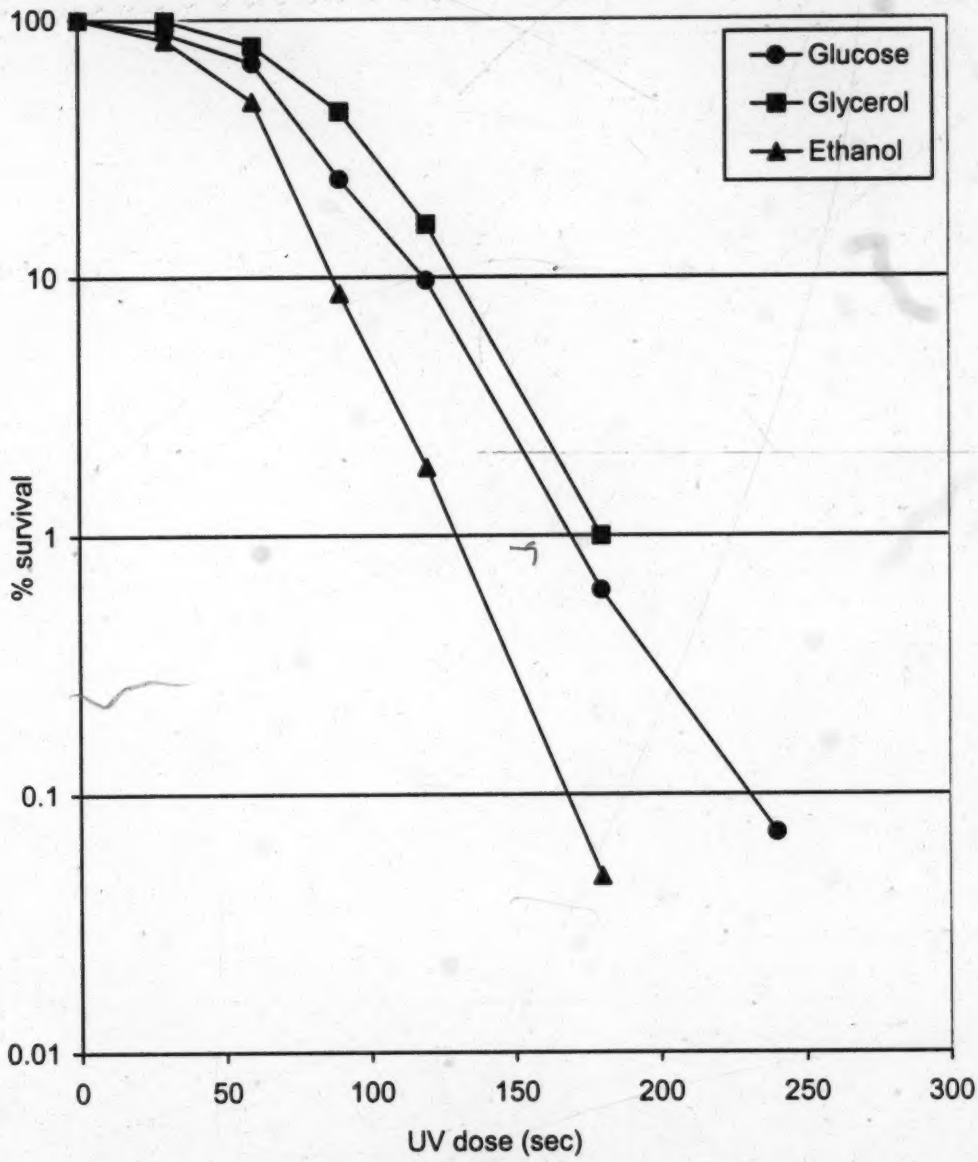
[a] Mutation rate = mutations/cell/generation.
[b] The strains were contructed in and are isogenic to JA6.
[c] The *rag5* mutation in *K. lactis* is homologous to the *hxk2* mutation in *S. cerevisiae*.

Table 13. Non-inducing strains of *Kluyveromyces lactis* have much higher spontaneous mutation rates on glucose than wildtype in the compartmentalization test.

| Carbon Source | Number of wells scored | Non-revertant wells | Cells per well ($x 10^6$) | Mutation rate[a] ($x10^8$) |
|---|---|---|---|---|
| *fog1* [b,c] | | | | |
| glucose | 422 | 22 | 4.71 | 31.3 |
| glycerol | 381 | 293 | 3.91 | 3.35 |
| *fog2* [b,d] | | | | |
| glucose | 406 | 29 | 10.0 | 13.1 |
| glycerol | 350 | 204 | 7.29 | 3.70 |
| *fog1 SFG*[c] | | | | |
| glucose | 234 | 55 | 8.13 | 8.91 |
| glycerol | 558 | 143 | 31.4 | 2.16 |

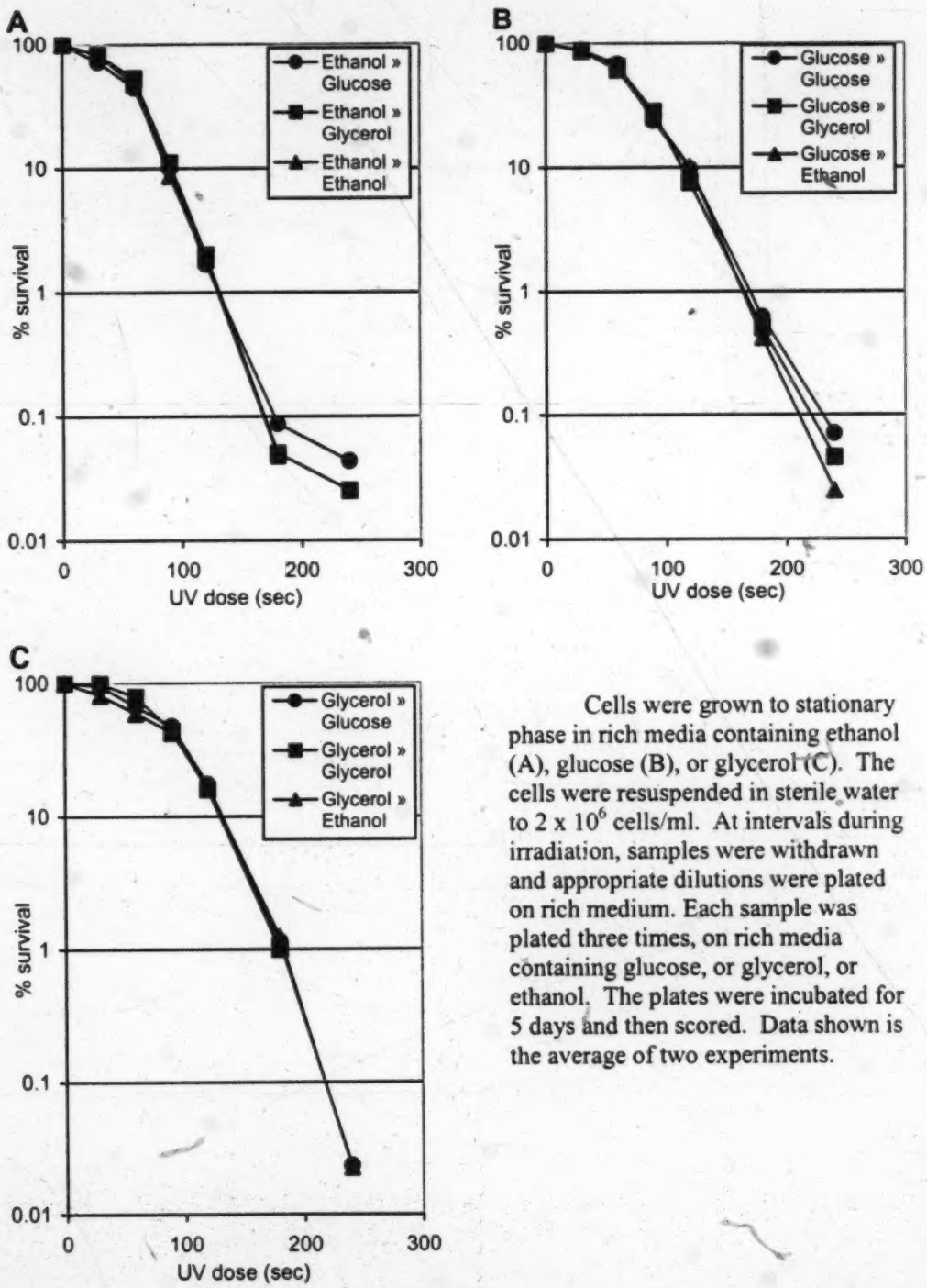
[a] Mutation rate = mutations/cell/generation
[b] The strains were contructed in and are isogenic to JA6.
[c] The *rag5* mutation in *K. lactis* is homologous to the *hxk2* mutation in *S. cerevisiae*.
[d] The *rag5* mutation in *K. lactis* is homologous to the *hxk2* mutation in *S. cerevisiae*.

Figure 2. Ethanol-grown *K. lactis* strain JA6 is more sensitive to UV irradiation.



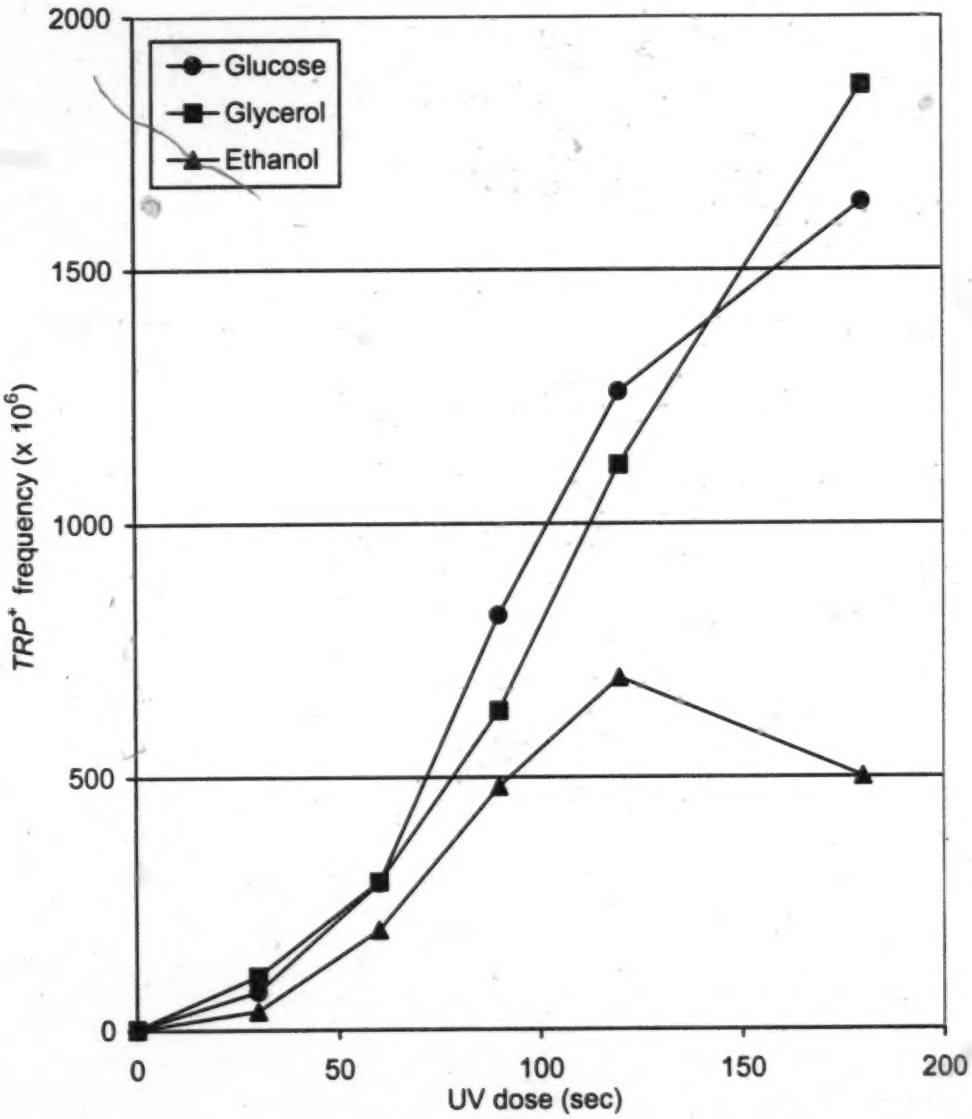

Cells were grown to saturation in rich media. Cells were resuspended in sterile water to 2 x $10^6$ cells/ml and irradiated as indicated. Following UV exposure, the cells were diluted in sterile water and plated on rich media containing the same carbon source as present during the pregrowth period. UV fluence was measured as 9.0 ergs/mm/sec. Data shown is the average of three experiments.

Figure 3. UV sensitivity in *K. lactis* strain JA6 correlates with pre-irradiation growth medium, not post-irradiation growth medium.



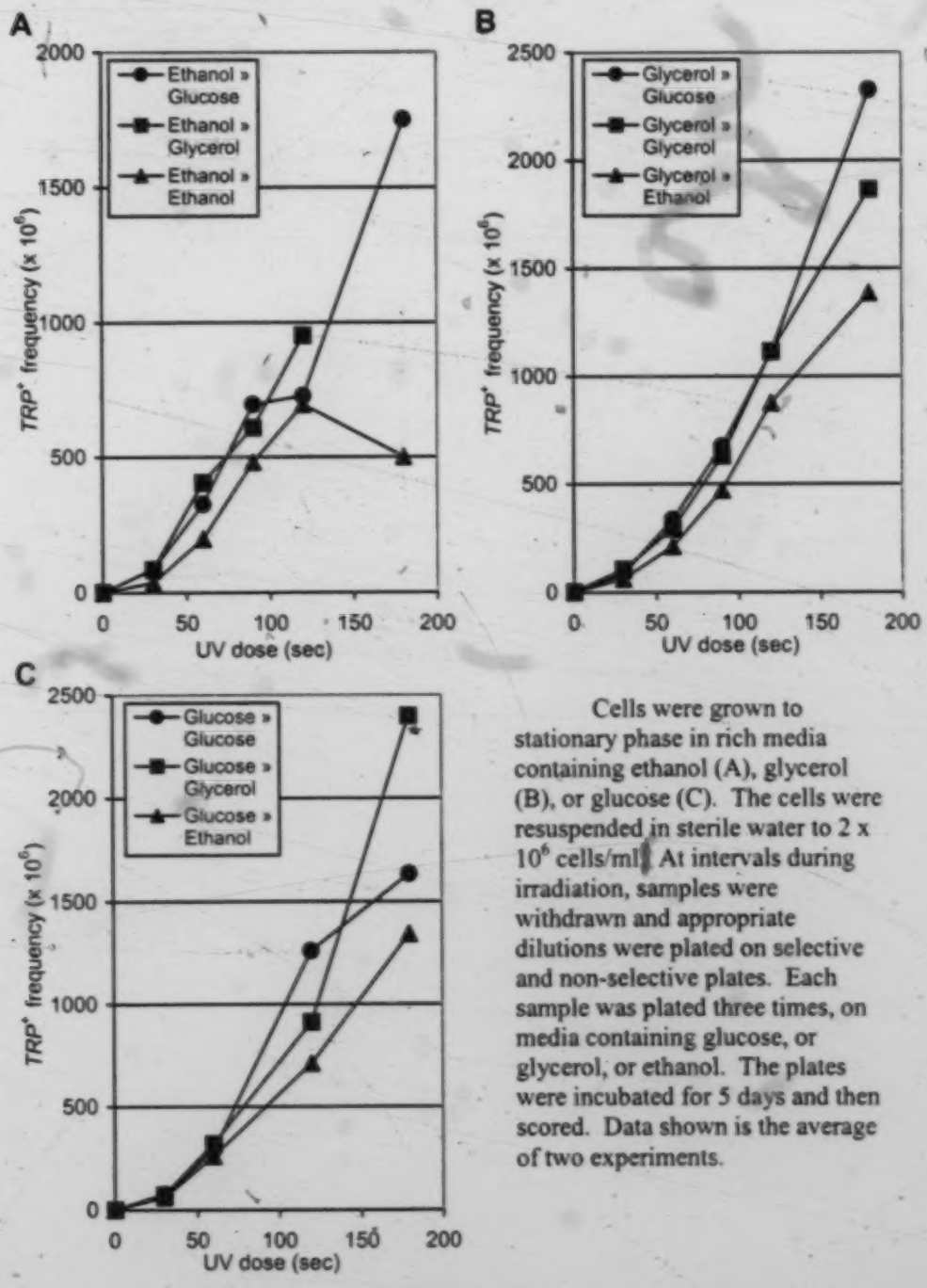

Cells were grown to stationary phase in rich media containing ethanol (A), glucose (B), or glycerol (C). The cells were resuspended in sterile water to 2 x $10^6$ cells/ml. At intervals during irradiation, samples were withdrawn and appropriate dilutions were plated on rich medium. Each sample was plated three times, on rich media containing glucose, or glycerol, or ethanol. The plates were incubated for 5 days and then scored. Data shown is the average of two experiments.

Figure 4. UV-induced reversion of *trp1-11* is limited at high doses in ethanol-grown cultures of *K. lactis*.



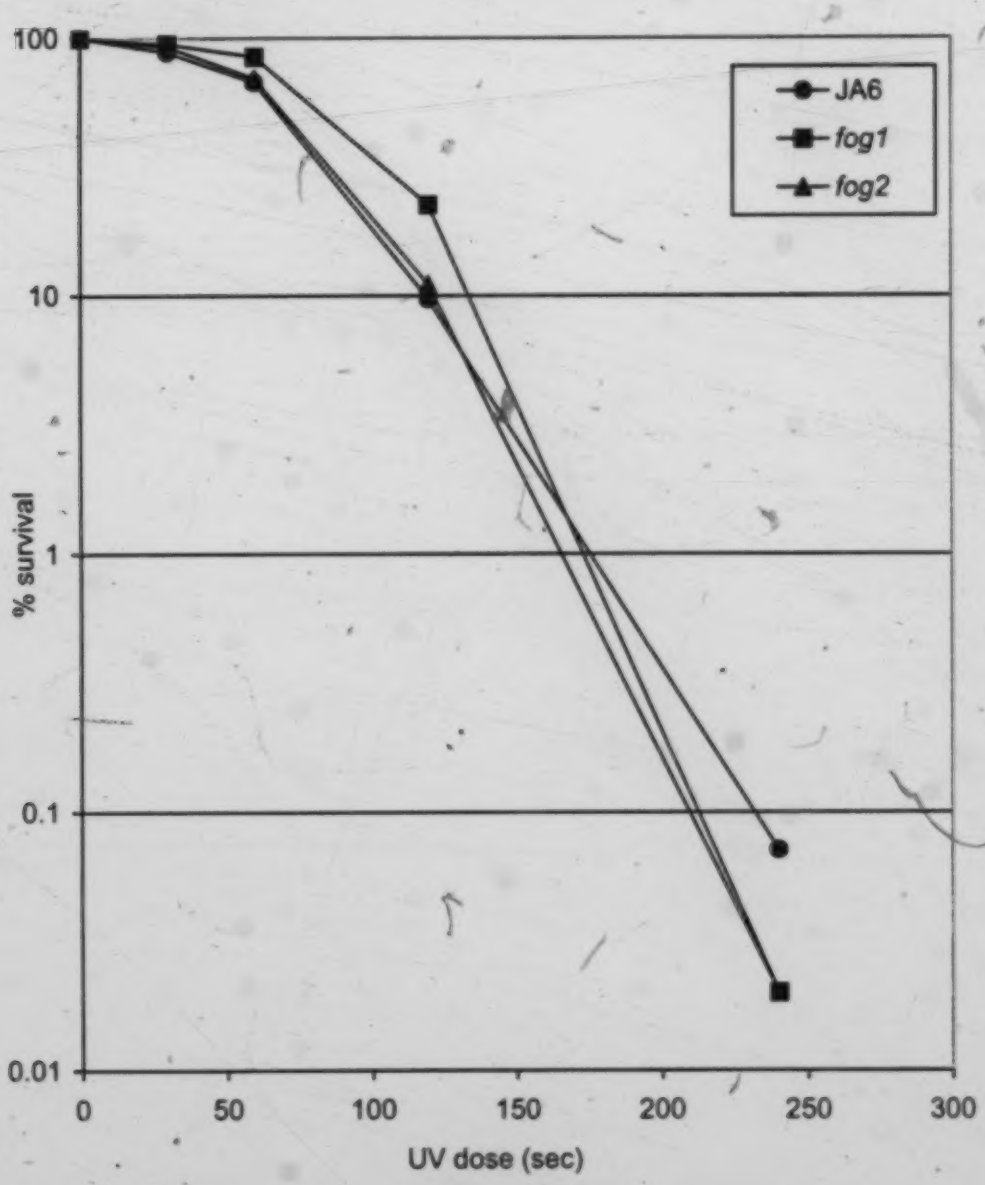

Cells were grown to saturation in rich media, then resuspended in sterile water to 2 x $10^6$ cells/ml and irradiated as indicated. At intervals, the cells were diluted in sterile water and appropriate dilutions were spread on selective and non-selective plates containing the same carbon source as present during the pregrowth period. UV fluence was measured as 9.0 ergs/mm/sec. Data shown is the average of two experiments.

Figure 5. The presence of glucose or glycerol during post-irradiation recovery increases the recovery of *K. lactis trp1-11* revertants from ethanol-grown cultures at high UV doses.



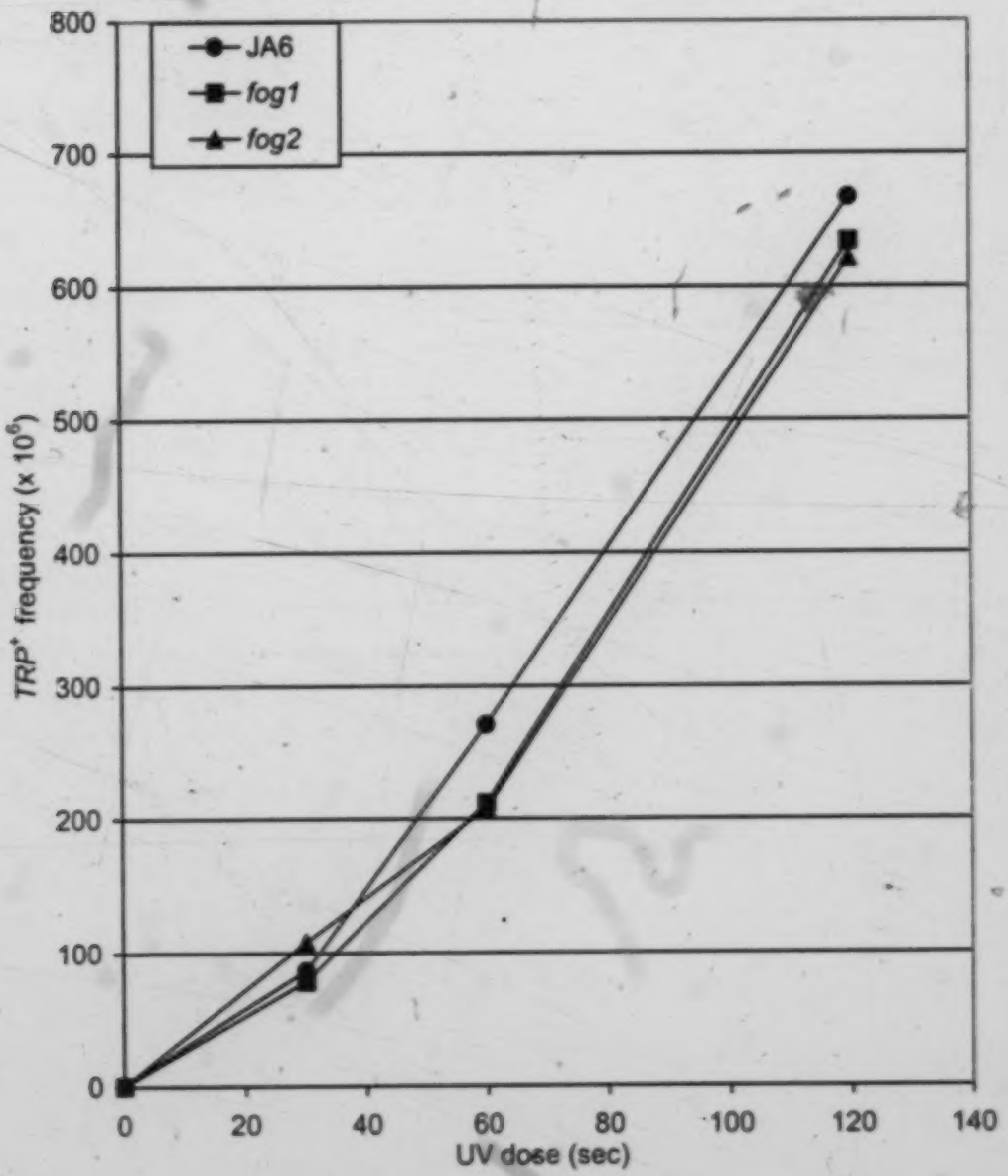

Cells were grown to stationary phase in rich media containing ethanol (A), glycerol (B), or glucose (C). The cells were resuspended in sterile water to 2 x $10^6$ cells/ml. At intervals during irradiation, samples were withdrawn and appropriate dilutions were plated on selective and non-selective plates. Each sample was plated three times, on media containing glucose, or glycerol, or ethanol. The plates were incubated for 5 days and then scored. Data shown is the average of two experiments.

Figure 6. UV survival is not affected in *fog1* and *fog2* strains of K. lactis.



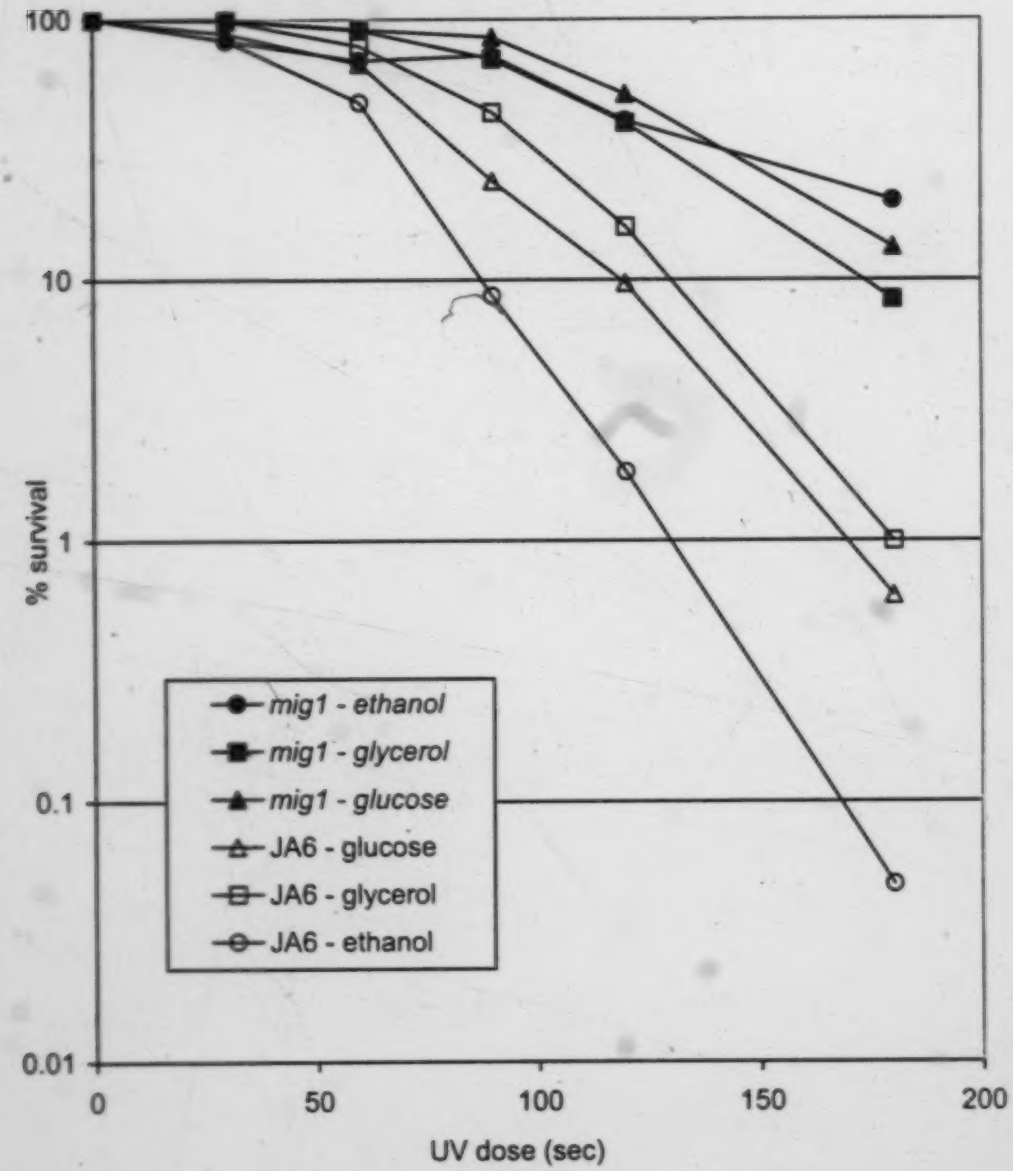

Cells were grown to saturation in rich media. Cells were resuspended in sterile water to 2 x $10^6$ cells/ml and irradiated as indicated. Following UV exposure, the cells were diluted in sterile water and plated on rich media containing the same carbon source as present during the pregrowth period. UV fluence was measured as 9.0 ergs/mm/sec. Data shown is the average of two experiments.

Figure 7. UV-induced reversion of *trp1-11* is unaffected by *fog* mutants in *K. lactis*.



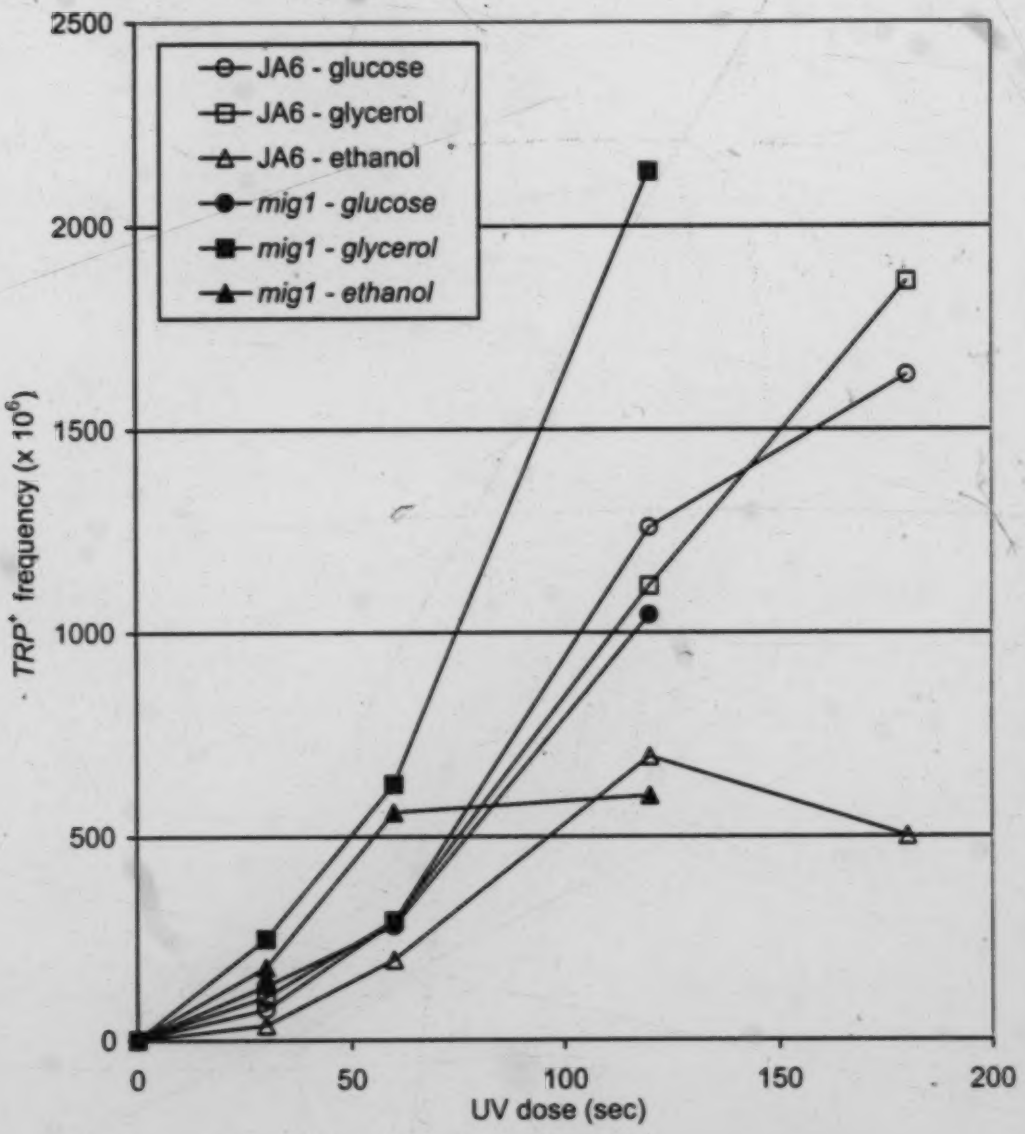

Cells were grown to saturation in rich media, then resuspended in sterile water to $2 \times 10^6$ cells/ml and irradiated as indicated. At intervals, the cells were diluted in sterile water and appropriate dilutions were spread on selective and non-selective plates containing the same carbon source as present during the pregrowth period. UV fluence was measured as 9.0 ergs/mm/sec. Data shown is the average of two experiments.

Figure 8. *mig1* increases survival of *K. lactis* following UV irradiation.




Cells were grown to saturation in rich media. Cells were resuspended in sterile water to 2 x $10^6$ cells/ml and irradiated as indicated. Following UV exposure, the cells were diluted in sterile water and plated on rich media containing the same carbon source as present during the pregrowth period. UV fluence was measured as 9.0 ergs/mm/sec. Data shown is the average of three experiments.

Figure 9. *K. lactis mig1* strains show an increased UV-induced reversion frequency in glycerol or ethanol cultures.




Cells were grown to saturation in rich media, then resuspended in sterile water to $2 \times 10^6$ cells/ml and irradiated as indicated. At intervals, the cells were diluted in sterile water and appropriate dilutions were spread on selective and non-selective plates containing the same carbon source as present during the pregrowth period. UV fluence was measured as 9.0 ergs/mm/sec. Data shown is the average of two experiments for the JA6 results and three experiments for the *mig1* results.

Table 14. Excision repair and recombination repair interact with carbon source to synergistically reduce spontaneous mutation rates in *Saccharomyces cerevisiae* on non-glucose carbon sources.

| Carbon source | Number of compartments | Number of non-mutant compartments | Background mutants/compt | Cells/ compartment ($\times 10^{-6}$) | Mutation rate ($\times 10^{8}$) |
|---|---|---|---|---|---|
| **Excision Repair** | | | | | |
| Y0418-5D *rad3-12* | | | | | |
| glucose | 618 | 438 | 0.016026 | 3.68 | 44.6 |
| glycerol | 623 | 618 | 0.016026 | 2.70 | <0.03 |
| ethanol | 615 | 590 | 0.016026 | 3.59 | 3.55 |
| **Recombination Repair** | | | | | |
| HP152-8A *mut5 (rad51)* | | | | | |
| glucose | 592 | 228 | 0.226763 | 3.40 | 106.0 |
| glycerol | 559 | 366 | 0.226763 | 2.80 | 35.1 |
| ethanol | 518 | 415 | 0.226763 | 1.50 | <0.73 |
| **Mutagenic Repair** | | | | | |
| Y0151-2D *rad6* | | | | | |
| glucose | 620 | 555 | 0.003205 | 2.89 | 18.6 |
| glycerol | 614 | 611 | 0.003205 | 2.50 | 0.34 |
| HP186-8C *rad18* | | | | | |
| glucose | 538 | 399 | 0.002804 | 2.50 | 59.2 |
| glycerol | 489 | 425 | 0.002804 | 1.70 | 40.4 |
| ethanol | 484 | 473 | 0.002804 | 1.00 | 10.1 |

[a] Mutation rate = mutations/cell/generation

Table 15. Some strains of *Saccharomyces cerevisiae* do not exhibit a carbon-source-dependent alteration of spontaneous mutation rate.

| Carbon source | Number of compartments | Number of non-mutant compartments | Background mutants/compt. | Cells/ compartment ($\times 10^{-6}$) | Mutation rate[a] ($\times 10^{-8}$) |
|---|---|---|---|---|---|
| **Wild-Type Repair** | | | | | |
| XV2023-11A | | | | | |
| glucose | 620 | 563 | 0.002804 | 2.00 | 23.4 |
| glycerol | 557 | 494 | 0.002804 | 2.00 | 29.3 |
| ethanol | 567 | 519 | 0.002804 | 2.00 | 21.4 |
| **Excision Repair** | | | | | |
| XV363-1A *rad 1* | | | | | |
| glucose | 503 | 412 | 0.002804 | 3.00 | 32.8 |
| glycerol | 454 | 367 | 0.002804 | 3.00 | 35.0 |
| ethanol | 619 | 583 | 0.002804 | 1.40 | 20.4 |
| **Mutagenic Repair** | | | | | |
| XV2023-8C *rev3* | | | | | |
| glucose | 510 | 480 | 0.0016 | 2.72 | 1.09 |
| glycerol | 562 | 520 | 0.0016 | 2.67 | 1.42 |
| ethanol | 592 | 570 | 0.0016 | 2.49 | 0.73 |

[a] Mutation rate = mutations/cell/generation

## Discussion

The effect that changing carbon source has on spontaneous mutagenesis was unexpected at the beginning of this work. Spontaneous mutation has been considered to be a natural property of a cell and treated as a static characteristic. This approach simplified analysis and theoretical discussion of the processes leading to spontaneous mutation by allowing investigators to focus on DNA injury and metabolism. The environmental context of the cell was rarely considered, unless it was known to lead to the generation of a direct damaging effect upon the DNA.

The results that I present in this thesis do not allow that simplified perspective to persist. My observations show that carbon metabolism has a large effect on spontaneous mutation rates, as much as 9-fold less in ethanol-grown cultures of *S. cerevisiae* and 30-fold higher in acetate-grown *K. lactis* cultures. These effects are larger than the changes in spontaneous mutation rate observed in strains carrying some DNA repair deficiencies (Haynes and Kunz, 1981). The study of spontaneous mutagenesis now must include the metabolic and developmental state of the cell in its explanations of cause and effect.

When *Saccharomyces cerevisiae* is grown on ethanol, the spontaneous mutation rate drops by a factor of nine (Table 8). One possible explanation of this phenomenon, is the production of antioxidant defenses when the mitochondria become active. Indeed, there are several antioxidant enzymes and compounds which increase in abundance when the mitochondria become active (Piper, 1995). However, induction of the mitochondria also occurs when yeast cells are grown on glycerol or xylose, but the change in mutation rate that is observed is only 3-fold with these carbon sources. This observation does not

support the hypothesis of induction of protection against oxidizing chemicals emanating from the mitochondria, such as singlet oxygen, superoxide, and free radicals.

The carbon sources tested on *S. cerevisiae* (Table 8) can be sorted into three groups based on mutation rate similarity. Glucose, sucrose, and fructose all have high mutation rates. These carbon sources are preferred by the cell, and are commonly encountered in natural settings. Interestingly, sucrose requires the release of glucose repression for the expression of invertase (Carlson, 1981), yet still exhibits a mutation rate similar to the glucose culture. Galactose, glycerol, and xylose all show mutation rates in *Saccharomyces cerevisiae* that are 3-fold lower than the first group. Ethanol and maltose exhibit very low mutation rates. There appears to be an overall correlation in *S. cerevisiae* between lower mutation rates and less preferred carbon sources. Growth is generally retarded in media which contains a less-favored carbon source. One possible explanation would propose that as the rate of growth of the cell decreases, due to the utilization of a non-favorable carbon source, the spontaneous mutation rate also decreases. Previous work in our lab has shown that the reduced rate of growth observed in glycerol cultures can be compensated by the inclusion of ethanol with the glycerol, allowing growth rates similar to glucose cultures (von Borstel, personal communication). Mutation rates for mixed glycerol/ethanol cultures are the same as glycerol alone, despite the growth rate difference (Table 8), indicating that the change in mutation rate is not a growth-rate-dependent phenomenon.

The carbon source effect is not specific to one type of mutation. The effect is present for *ura3-13*, a 23 bp duplication (Lee *et al.*, 1988), *his1-7*, a missense mutation which is able to revert at many sites (von Borstel, 1998), and *arg4-17*, an ochre mutant

which reverts at the locus only by transversion, and can be suppressed by mutations in several tRNA genes (Gottlieb and von Borstel, 1975). All of these markers show the carbon source effect, though to different degrees (Table 9). The largest effects are seen for the *ura3-13* duplication and the tRNA suppressors of the *arg4-17* ochre mutant.

Sequencing of the *HIS*$^+$ revertants does not appear to show any specific preference for a particular mutational change, rather all types of base substitutions are reduced (M. Chrenek, personal communication). These observations argue against the possibility that the change in carbon source generates (or removes) a mutagenic metabolic intermediate. Were such an intermediate present, the lesion(s) generated would be specific, affecting one specific type of mutation, much as the mutational spectra for nearly all known mutagens (Kunz *et al.*, 1998). This does not appear to be the case. Unless we include a change in a large number of mutagenic compounds with a variety of targets, this model is untenable.

The effect of changes in carbon metabolism on spontaneous mutation we observe in *Saccharomyces cerevisiae* is also observed in a related yeast, *Kluyveromyces lactis*. The spontaneous mutation rate is very different for glucose grown cultures versus ethanol grown cultures. However, there is the very obvious difference that the direction of mutation rate change is reversed. In *Kluyveromyces lactis*, the higher mutation rate is observed in ethanol-grown cultures (Table 10) in contrast to the low rate of spontaneous mutation observed in *Saccharomyces cerevisiae* strains. When we compare the effects of several different carbon sources on mutation rates in the two species, it becomes apparent that there is a commonality between the two yeasts. Despite the differences in the specific effects of the change in carbon source on mutation rate, the ordering of the

carbon sources by their spontaneous mutation rate relative to glucose in the two species is very similar. The carbon sources that are most like glucose are sucrose, fructose and lactose[2]. Glycerol, galactose and xylose have intermediate effects upon the spontaneous mutation rate in both species, and ethanol and maltose have the most extreme effects. This strongly suggests that the mechanism by which the spontaneous mutation rate is being affected is evolutionarily conserved, and therefore must have a genetic basis.

The dependence of spontaneous mutation rate on carbon source suggests that there is a connection to carbon metabolism. At least part of the connection between mutation rate and carbon source is mediated by carbon regulation. The investigation of the carbon regulation was initially carried out in *Kluyveromyces lactis*. *K. lactis* has a much simpler regulatory system which allows single-mutant analysis of the interaction between carbon regulation and spontaneous mutation (Iliana Ferrero, personal communication; Goffrini *et al.*, 1996).

The carbon regulation system is composed of an alternating series of positive and negative regulators of protein function which begins with hexokinase II (*HXK2*) and terminates in a transcriptional repressor encoded by the *MIG1* gene. Deletion of *MIG1* releases all glucose-repressed genes, making them capable of expression. Most genes require the additional presence of a specific carbon source for full expression (Gancedo, 1998).

In order to permit expression of the genes necessary for utilization of alternate carbon sources, the Mig1 protein must be inactivated. The rate of reversion of *trp1-11* in *Kluyveromyces lactis* increases when non-glucose carbon sources are used. The higher

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[2] *Saccharomyces cerevisiae* is unable to utilize lactose as a carbon source due to the absence of a β-galactosidase.

mutation rate observed on non-glucose carbon sources in *Kluyveromyces lactis* may be due to the inactivation of *MIG1*-mediated repression. We would expect that the mutation rate on glucose would therefore increase in a *Δmig1* strain. Contrary to expectations, the *mig1* mutation did not cause an increase in mutation rate on glucose. Instead, the spontaneous mutation rates for glycerol, maltose and ethanol-grown cultures declined. A similar effect was observed when a hexokinase mutant was tested, confirming that the reduction in mutation rate is due to a release of catabolite repression.

A same effect was observed in *Saccharomyces cerevisiae mig1* and *hxk2* strains (Table 11). In the *S. cerevisiae mig1* strain, there is a very pronounced negative effect upon spontaneous mutation rate in ethanol or maltose cultures. A lesser effect is observed for glycerol cultures and little effect upon glucose cultures. The *hxk2* strain exhibits the same pattern, though to a lesser extent for ethanol and maltose. The similarity of the observations in *Kluyveromyces lactis* and *Saccharomyces cerevisiae* strongly implies a conserved regulatory mechanism that controls not only carbon metabolism, but spontaneous mutation as well.

The Mig1 protein is regulated directly by the Snf1/4 kinase complex, which is necessary for expression of glucose-repressed genes. The Snf complex also has positive expression effects which are not related to the Mig1 repression (I. Ferrero, personal communication; Lesage, *et al.*, 1996; Hu *et al.*, 2000). Deletion of the *SNF1* gene results in an inability to grow on carbon sources other than glucose or fructose (Carlson, 1987).

Snf1 kinase function in *K. lactis* is encoded by the *FOG1* and *FOG2* genes (Goffrini, *et al.*, 1996). The *FOG2* gene encodes the kinase component (*Sc-SNF1*) and the *FOG1* gene encodes a protein associated with the kinase which appears to be

important for target selection. In *S. cerevisiae*, there are at least four homologs of the *FOG1* gene (Jiang and Carlson, 1997), preventing ready analysis of the mutant. When *fog1* and *fog2* mutants were tested for reversion of the *trp1-11* allele, both strains exhibited a strong mutator effect when grown on glucose, 15-fold for *fog1*, 7-fold for *fog2* (Table 13). The *fog* strains were capable of slow growth on glycerol as well, and exhibited a barely significant mutator effect on this medium. This observation suggests that the inactive form of the kinase complex has a specific role in the prevention of mutation.

A strain carrying a suppressor of the *fog1* mutation was isolated which allowed the *fog1* strains to grow on other carbon sources. When the spontaneous mutation rate was tested in this strain, it was found to retain mutator activity on glucose, but at much reduced level, and the glycerol mutation rate dropped as well. This strain appears to exhibit the phenotype expected by a combination of the *mig1* and *fog1* phenotypes, if they affected different regulatory pathways. However, a known *Δmig1 Δfog1* strain is unable to grow on non-glucose carbon sources (P. Goffrini, personal communication), indicating that the suppressor must be either in a separate pathway, or a different member of the regulatory pathway.

The *snf1* mutant in *Saccharomyces cerevisiae* did not confer a mutator phenotype (Table 11). The *K. lactis* homolog, *fog2*, has a lesser effect than *fog1*, which may indicate that the activity of the *fog1* gene product is most important, and the *fog2* gene product serves a lesser role which is not an absolute requirement for the *fog1* effect. As mentioned above, the *fog1* gene has multiple homologs in *Saccharomyces cerevisiae*. One of the *fog1* homologs in *S. cerevisiae*, *gal83*, has no phenotype as a deletion mutant.

The gene was originally identified as a dominant allowing escape from glucose repression. Overexpression of *SIP1* or *SIP2* can suppress the dominant *GAL83-2000* allele that relieves glucose repression of the *GAL* operon (Erickson and Johnston, 1993). Together, the additional homologs in *S. cerevisiae* may retain enough function to suppress the expected mutator phenotype in this species.

The changes in spontaneous mutation rate that occur when the carbon regulatory system is altered in both yeast species confirm the involvement of carbon metabolism in the determination of the spontaneous mutation rate. The effects observed in *K. lactis*, and supported by the *S. cerevisiae* results suggest that there are at least two separate points of influence, one effected by the Mig1 repressor, and another operating through the Snf1 kinase, independent of Mig1 function.

The changes in spontaneous mutation rate could be due to a change in the overall ability of the cell to handle DNA damage. This change should alter the ability of the cell to respond to induced damage. This was tested using ultraviolet light, which allows the involvement of excision repair and recombination repair to be evaluated. The growth of *Kluyveromyces lactis* on ethanol caused an increased sensitivity to UV light (Fig 2). This was not correlated with an increase in induced mutation frequency, but a limitation on the extent of induced mutation at higher doses (Figure 4). The induction of *trp1-11* revertants does not increase beyond 600 per $10^6$ survivors, observed at 120s of UV exposure. The reduction in survival could not be rescued by plating ethanol-grown cultures on glucose or glycerol (Fig 3), but the limitation on mutation induction was rescued by plating on glucose or glycerol (Fig 5). Again, this suggests that two different regulatory functions are active in modifying mutation rates.

The changes in repair capacity are due to regulatory changes. The survival defect observed for cultures grown on ethanol can be rescued by a mutation in *mig1* (Figure 8), indicating that the change in DNA repair is due to a change in transcriptional regulation. The presence of *mig1* also leads to a general increase in UV-induced *trp1* reversion frequencies, though the limit on mutation that is observed in ethanol cultures is still present. The *mig1* effect on mutation frequency is not expected, and suggests that the Mig1 repressor must still have a function even when unable to bind to DNA. The Mig1 protein mediates its repressive effect through interaction with the Ssn6-Tup1 repressor complex (Treitel and Carlson, 1995), which is involved with the repression of many genes through interaction with specific targeting proteins (Keleher *et al.*, 1992). It is possible that the lack of functional Mig1 protein affects the cellular distribution of the Ssn6-Tup1 complex, and affects the repression activity at other gene complexes. This hypothesis can be tested by employing a *mig1* allele that still produces a protein, but one that is unable to bind to the *MIG1* recognition site.

The altered UV response in ethanol-grown cells and in *mig1* cells of *Kluyveromyces lactis* indicates that the carbon effect on spontaneous and induced mutation rate must affect DNA repair. In addition, there may be specific aspects of DNA repair affected by each carbon source. The key enzymes of the major repair pathways were tested in *Saccharomyces cerevisiae*; *RAD3* for nucleotide excision repair; *RAD51* for recombination repair; and *RAD18* and *RAD6* for post-replication mutagenic repair. These genes interact synergistically with any mutant in the other repair pathways indicating that they are absolutely required for repair activity in their respective pathways (Cox and Game, 1971). In addition, *REV3*, which has been shown to be required for

mutation induction in several pathways, was tested (Glassner *et al.*, 1998; Holbeck and Strathern, 1997; Koval'tsova *et al.*, 1996; Roche *et al.*, 1995; Roche *et al.*, 1994).

The mutator effects which have been observed previously (von Borstel *et al.*, 1971; Brychcy and von Borstel, 1977; Morrison and Hastings, 1979; Quah *et al.*, 1980) in glucose cultures were also observed in my experiments (Table 14). Overall, the mutator activity persisted in the glycerol and ethanol-grown cultures, but continued to show the carbon source effect. Spontaneous mutation rates in ethanol cultures were less than rates in glycerol cultures, and glycerol cultures had spontaneous mutation rates that are less than rates in glucose cultures. The *rad6* strain showed a more extreme decrease in spontaneous mutation rate on glycerol than the rest of the strains. In contrast, the *rad18* mutant shows roughly the same enhancement of mutation rate on all three-carbon sources, despite being a member of the same epistasis group as *rad6*, suggesting a role for rad6 in determining the spontaneous mutation rate. The *rev3* mutant shows the expected reduced spontaneous mutation rate on glucose, and on glycerol and on ethanol.

Two strains showed sharp deviations from this pattern. The *rad3* mutant has no detectable spontaneous mutation events when grown on glycerol (Table 14). The *rad51* mutant shows a similar phenotype when grown on ethanol. Both situations show a reduced cell number in the compartments, which may be indicative of cells which are metabolically active, but are unable to progress in the cell cycle. It seems possible that the lack of newly arising revertants is due to unrepaired lesions that are preventing the cells from dividing. Potential revertants that would be produced by misrepair events are not produced due to the block to lesion repair. The results suggest that cells grown on glycerol require nucleotide excision repair for the repair of spontaneous lesions and the

generation of spontaneous mutations. Similarly, in ethanol-grown cells the double-strand-break repair system is required for repair and mutagenesis. In both cases, the majority of the spontaneous mutagenesis observed must also require processing by the Rev3/Rev7 mutagenic polymerase ζ, which eliminates the majority of spontaneous mutation events in this system.

Channeling of lesions has been suggested previously as an explanation for the mutator activity observed when a particular repair defect is present (Hastings *et al.*, 1976), positing that the repair of spontaneous lesions is undertaken by other pathways when the preferred pathway is absent. These pathways are more prone to errors than the preferred pathway, which generates an increased rate of spontaneous mutagenesis. My observations require the additional sequestering of the lesions when there is a non-glucose carbon source present, preventing the other pathways from accessing the lesion, or limiting error-prone repair to one specific repair pathway. The channeling observed here has the effect of limiting errors.

Two strains, XV2023-11A and HP363-1A, were identified which lack the carbon source associated change in mutation rate (Table 15). Both strains showed a slight mutator phenotype on glucose, but do not show the 3-fold decline in mutation rate on glycerol or the 9-fold decline on ethanol. Strain XV2023-11A was crossed to XV185-6A, but the resulting diploid was unable to produce complete tetrads for analysis. The diploid did show the carbon source effect in Lassie tests, as did the haploid parent XV185-6A, indicating that the carbon source effect is the dominant trait.

These two strains must carry a mutation in a gene or genes which connect DNA metabolism to carbon regulation, a gene I have tentatively named *CSM1* (catabolite

regulation of spontaneous mutation). Strain XV2023-11A is a repair-proficient strain, which is not UV-sensitive; HP363-1A carries an excision repair mutation (*rad1*), but does not show the decline in mutation rate in glycerol culture described above for *rad3*, suggesting that the unknown mutant is epistatic to the excision repair-glycerol synergism.

Modeling the carbon effect on spontaneous mutation.

The results presented here are indicative of a defined, evolutionarily conserved regulatory pathway which links carbon metabolism to DNA repair. When glucose is present, the glucose is phosphorylated by Hxk2, generating the signal that inactivates the Snf1 kinase, preventing the inactivation of Mig1. The unphosphorylated Mig1 repressor blocks expression of carbon-regulated genes, which includes *CSM1*, which prevents modulation of DNA repair, allowing full access of DNA repair to all lesions. When an alternate carbon source is present, the Snf1 kinase becomes active and inactivates Mig1, releasing the repression of the carbon regulated genes. The *CSM1* gene product then modulates DNA repair and, ultimately, this affects spontaneous mutation rates. Once the repair of the lesion is underway, there is a finite probability that the repair will be erroneously finished by the Rev3/7 mutagenic polymerase. The changes in spontaneous mutation rate observed when carbon source is changed come about due to the variable chance of producing a substrate for the Rev3/7 polymerase during repair by different repair systems.

Future directions.

Several questions remain open at this stage of investigation into this phenomenon. The identity of the *CSM1* needs to be determined. Attempts to analyze the inheritance of the marker failed due to sporulation difficulties. The allele present in XV2023-11A appears to be recessive to that of the XV185-14C allele (not shown), suggesting that the modulating phenotype is the wildtype characteristic. The mechanism by which the *CSM1* gene product generates its effect needs to be determined as well. Investigation of a broader range of DNA damaging agents may better define the actual changes in the DNA repair which are occurring. Expression profiling which compares active growth on glucose to active growth on other carbon sources may permit identification of the genes that are being affected. Current studies on carbon utilization and regulation in wildtype cells have only investigated the change in expression that occurs during the diauxic shift which occurs following glucose exhaustion (Derisi, *et al.*, 1997). Only *RAD52* (2-fold decrease) and *RAD4* (2-fold increase) were significantly affected by the transition. This small change does not seem enough to cause the shifts in mutation rate I have observed. An investigation of the Mig1/Mig2 repressors did not identify any DNA repair genes as among the genes that are affected by Mig repression (Lutfiyya, *et al.*, 1998). In both studies, a large number of genes with no identified function were affected.

Further dissection of the carbon regulation genes that affect DNA repair would help identify the probable mechanism which transmits the signal. There are multiple regulatory pathways that respond to the carbon source (Thevelein and Hohmann, 1996).

Overlaps with stress response systems (Blomberg, 1999) and mitochondria-nucleus signaling (Kirchman *et al.*, 1999) may also influence the DNA repair response of the cell.

The differences and similarities in the response of spontaneous mutagenesis to changes in carbon source in *S. cerevisiae* and *K. lactis* needs to be investigated. strains carrying the *mig1* mutation behaved similarly in both species, suggesting that some aspects of the carbon source-spontaneous mutagenesis connection are conserved, but the opposite pattern of mutagenesis levels on different carbon sources suggests that some great differences exist. One possible source of these differences is the petite-negative characteristic of *K. lactis*. This species must have a functional mitochondria present, regardless of carbon source. In *S. cerevisiae*, the mitochondria are active only as needed. The differences we see may be a result of the increased activity of the mitochondria, possibly due to differential activity against oxidative lesions. It is possible to create a petite strain of K. lactis (Chen and Clark-Walker, 1993). The behavior of this strain under our conditions may provide some insight into the differences observed between the two species.

Implications

The possible regulation of DNA repair by carbon metabolism suggests that the influence of other cellular responses to outside influences may dramatically affect the stability of the DNA. Heat shock, and osmotic shock are already known to cause induction of some DNA repair genes (Mitchel and Morrison, 1983, 1986; Mager, 1993). At the unicellular level, there are limited possibilities for more sophisticated regulation,

but in more advanced eukaryotes, there may be tissue specific effects upon DNA repair which will become important in the study of cancer biogenesis. It is already known that mismatch repair defects are common deficiencies in many tumors. However, inherited defects in mismatch repair lead to an increase in cancer incidence in a limited number of tissues (Lynch and de la Chapelle, 1999), primarily the distal colon. Further, mouse models of mismatch repair defects have a very different list of affected organs (Heyer *et al.*, 1999).

Conversely, DNA damaging chemicals act in a tissue specific manner. Smoking has been known as a major predisposing factor in the development of lung and bladder cancer. It is also an anticarcinogen agent for the development of colon cancer ( *cf* von Borstel and Higgins, 1998). An ongoing series of studies on the toxicity of various chemicals has shown that all compounds which exhibit a carcinogenic effect only affect specific tissues, not all cell types universally (Johnson, 1999). Unpublished results from the same set of studies indicate that most compounds have both mutagenic and antimutagenic activities, which vary by the tissue affected (F. M. Johnson, personal communication). Very few chemicals are solely mutagenic, or solely anti-mutagenic. The only reasonable explanation for the tissue-specific effects on mutagenesis is tissue-specific prevention or repair of DNA damage.

I have demonstrated that regulatory changes in general metabolism can have large effects upon the aspects of DNA metabolism that affect spontaneous mutation rates. These effects appear to be directed by a novel signaling pathway which alters DNA repair in response to carbon source.

## References


Ahn, B. Y., Dornfeld, K. J., Fagrelius, T. J. and Livingston, D. M. (1988). Effect of limited homology on gene conversion in a *Saccharomyces cerevisiae* plasmid recombination system. *Molecular and Cellular Biology* **8,** 2442-2448.

Anson, R. M., Hudson, E. and Bohr, V. A. (2000). Mitochondrial endogenous oxidative damage has been overestimated. *Federation of American Societies for Experimental Biology (FASEB) Journal* **14,** 355-360.

Atwood, K. (1953). Postulation of a checking system during DNA replication. *Meeting at the Oak Ridge National Laboratory held to examine possible reasons for the high accuracy of DNA replication. Washington, D.C.*

Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F. and Cullin, C. (1993). A simple and efficient method for direct gene detection in *Saccharomyces cerevisiae*. *Nucleic Acids Research* **21,** 3329-3330.

Blomberg, A. (2000). Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. *FEMS Microbiological Letters* **182,** 1-8.

Bohme, H. (1967). Genetic instability of an ultraviolet-sensitive mutant of *Proteus mirabilis*. *Biochemical and Biophysical Research Communications* **28,** 191-198.

Boles, E. and Hollenberg, C. P. (1997). The molecular genetics of hexose transport in yeasts. *Federation of European Microbiological Societies (FEMS) Microbiological Reviews* **21,** 85-111.

Breunig, K. D. (1989). Multicopy plasmids containing the gene for the transcriptional activator *LAC9* are not tolerated by *K. lactis* cells. *Current Genetics* **15,** 143-148.

Brutlag, D. and Kornberg, A. (1972). Enzymatic synthesis of deoxyribonucleic acid. XXXVI. A proofreading function for the 3'-->5' exonuclease activity in deoxyribonucleic acid polymerases. *Journal of Biological Chemistry* **247,** 241-248.

Brychcy, T. and von Borstel, R. C. (1977). Spontaneous mutability in UV-sensitive excision-defective strains of *Saccharomyces*. *Mutation Research* **45,** 185-194.

Burnet, F. M. (1929). Smooth-rough variation in bacteria in its relation to bacteriophage. *Journal of Pathological Bacteria* **32,** 15-42.

Cairns, J., Overbaugh, J. and Miller, S. (1988). The origin of mutants. *Nature* **335,** 142-145.

Carlson, M. (1987). Regulation of sugar utilization in *Saccharomyces* species. *Journal of Bacteriology* **169,** 4873-4877.

Carlson, M., Osmond, B. C. and Botstein, D. (1981). Mutants of yeast defective in sucrose utilization. *Genetics* **98,** 25-40.

Cassart, J. P., Georis, I., Ostling, J., Ronne, H. and Vandenhaute, J. (1995). The *MIG1* repressor from *Kluyveromyces lactis*: cloning, sequencing and functional analysis in *Saccharomyces cerevisiae*. *Federation of European Biochemical Societies (FEBS) Letters* **371,** 191-194.

Cassart, J. P., Ostling, J., Ronne, H. and Vandenhaute, J. (1997). Comparative analysis in three fungi reveals structurally and functionally conserved regions in the Mig1 repressor. *Molecular and General Genetics* **255,** 9-18.

Clark-Walker, G. D. and Chen, X. J. (1996). A vital function for mitochondrial DNA in the petite-negative yeast *Kluyveromyces lactis*. *Molecular and General Genetics* **252,** 746-750.

Chen, X. J. and Clark-Walker, G. D. (1993). Mutations in *MGI* genes convert *Kluyveromyces lactis* into a petite-positive yeast. *Genetics* **133**,517-525.

Correns, C. (1900). G. Mendel's Regel über das Verhalten der Nachkommenschaft der Rassenbastarde. *Berichte der deutschen botanischen Gesellschaft* **18,** 158-168.

Cox, B. and Game, J. (1974). Repair systems in *Saccharomyces*. *Mutation Research* **26,** 257-264.

Crabtree, H. G. (1929). Observations on the carbohydrate metabolism of tumors. *Biochemical Journal* **23,** 536-545.

Darwin, C. (1859). *On the Origin of Species by Means of Natural Selection*. John Murray.

de Vries, H. (1900). Das Spaltungsgesetz der Bastarde. *Berichte der deutschen botanischen Gesellschaft* **18,** 83-90.

Demerec, M. (1937). Frequency of spontaneous mutations in certain stocks of *Drosophila melanogaster*. *Genetics* **22,** 469-478.

Demerec, M. (1945). Production of *Staphylococcus* strains resistant to various concentrations of penicillin. *Proceedings of the National Academy of Science* **31,** 16-24.

Demple, B. and Harrison, L. (1994). Repair of oxidative damage to DNA: Enzymology and biology. *Annual Review of Biochemistry* **63,** 915-948.

Derisis, J. L., Iyer, V. R. and Brown, P. O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278,** 680-686.

D'Herelle, F. (1926). *The Bacteriophage and Its Behavior.* Williams and Wilkins.

Drake, J. W., Allen, F., Forsgerg, S. A., Preparata, R. M. and Greening, E. O. (1969). Spontaneous mutation: genetic control of mutation rates in bacteriophage T4. *Nature* **221,** 1128-1132.

Drake, J. W., Charlesworth, B., Charlesworth, D. and Crow, J. F. (1998). Rates of spontaneous mutation. *Genetics* **148,** 1667-1686.

England, T. G., Jenner, A., Aruoma, O. I. and Halliwell, B. (1998). Determination of oxidative DNA base damage by gas chromatography-mass spectrometry. Effect of derivatization conditions on artifactual formation of certain base oxidation products. *Free Radical Research* **29,** 321-330.

Erickson, J. R. and Johnston, M. (1993). Genetic and molecular characterization of *GAL83*: its interaction and similarities with other genes involved in glucose repression in *Saccharomyces cerevisiae*. *Genetics* **135,** 655-664.

Esterbauer, H., Eckl, P. and Ortner, A. (1990). Possible mutagens derived form lipids and lipid precursors. *Mutation Research* **238,** 223-233.

Fasullo, M., Bennett, T., AhChing, P. and Koudelik, J. (1998). The *Saccharomyces cerevisiae RAD9* checkpoint reduces the DNA damage-associated stimulation of directed translocations. *Molecular and Cellular Biology* **18,** 1190-1200.

Friedberg, E. C. and Gerlach, V. L. (1999). Novel DNA polymerases offer clues to the molecular basis of mutagenesis. *Cell* **98,** 413-416.

Game, J. C. and Cox, B. S. (1971). Allelism test of mutants affecting sensitivity to radiation in yeast and a proposed nomenclature. *Mutation Research* **12,** 328-331.

Gancedo, J. A. (1998). Yeast carbon catabolite repression. *Microbiological and Molecular Biology Reviews* **62,** 334-361.

Gietz, R. D. and Schiestl, R. H. (1995). Transforming yeast with DNA. *Methods in Molecular and Cellular Biology* **5,** 255-269.

Glassner, B. J., Rasmussen, L. J., Najarian, M. T., Posnick, L. M. and Samson, L. D. (1998). Generation of a strong mutator phenotype in yeast by imbalanced base excision repair. *Proceedings of the National Academy of Science USA* **95,** 9997-10002.

Glatt, H. (1990). Endogenous mutagens derived from amino acids. *Mutation Research* **238,** 235-243.

Goffrini, P., Algeri, A. A., Donnini, C., Wesolowski-Louvel, M. and Ferrero, I. (1989). *RAG1* and *RAG2*: nuclear genes involved in the dependence/independence on mitochondrial respiratory function for growth on sugars. *Yeast* **5,** 99-106.

Goffrini, P., Ficarelli, A., Donnini, C., Lodi, T., Puglisi, P. P. and Ferrero, I. (1996). *FOG1* and *FOG2* genes, required for the transcriptional activation of glucose-repressible genes of *Kluyveromyces lactis*, are homologous to *GAL83* and *SNF1* of *Saccharomyces cerevisiae*. *Current Genetics* **29,** 316-326.

Gottlieb, D. J. and von Borstel, R. C. (1976). Mutators in *Saccharomyces cerevisiae*: *MUT1-1*, *MUT1-2* and *MUT2-1*. *Genetics* **83,** 655-666.

Gratia, A. (1921). Studies on the D'Herelle phenomenon. *Journal of Experimental Medicine 34:115.* **34,** 115-126.

Green, M. M. (1969). Controlling element mediated transposition of the white gene in *Drosophila melanogaster*. *Genetics* **61,** 429-441.

Green, M. M. and Shepherd, S. H. (1979). Genetic instability in *Drosophila melanogaster*: The induction of specific chromosome 2 deletions by MR elements. *Genetics* **92,** 823-832.

Halas, A., Batranowska, H., Policinska, Z. and Jachymczyk, W. J. (1997). Involvement of the *REV3* gene in the methylated base-excision repair system. Co-operation of two DNA polymerases, d and Rev3p, in the repair of MMS-induced lesions in the DNA of *Saccharomyces cerevisiae*. *Current Genetics* **31,** 292-301.

Hamilton, M. D. and von Borstel, R. C. (1996). Measuring yeast spontaneous mutation rates when grown in different carbon sources. *Environmental and Molecular Mutagenesis* **27,** 27.

Hanawalt, P. C. and Haynes, R. H. (1965). Repair replication of DNA in bacteria: Irrelevance of chemical nature of base defect. *Biochemical and Biophysical Research Communications* **19,** 462-467.

Harris, R. L., Feng, G., Ross, K. J., Sidhu, R., Thulin, C., Longerich, S. L. and Rosenberg, S. R. (1997). Mismatch repair protein MutL becomes limiting during stationary-phase mutation. *Genes and Development* **11,** 2426-2437.

Hastings, P. J., Quah, S. K. and von Borstel, R. C. (1976). Spontaneous mutation by mutagenic repair of spontaneous lesions in DNA. *Nature* **264,** 719-722.

Haynes, R. H. and Kunz, B. A. (1981). DNA repair and mutagenesis in yeast. in Strathern, J. N., Jones, E. W. and Broach, J. R. (Eds), *Molecular Biology of the Yeast Saccharomyces*, Cold Spring Harbour Press, pp. 371-413.

Heyer, J., Yang, K., Lipkin, M., Edelmann, W. and Kucherlapati, R. (1999). Mouse models for colorectal cancer. *Oncogene* **18,** 5325-5333.

Hill, R. F. (1958). A radiation-sensitive mutant of *E. coli*. *Biochimica Biophysica Acta* **30,** 636-637.

Holbeck, S. L. and Strathern, J. N. (1997). A role for *REV3* in mutagenesis during double-strand break repair in *Saccharomyces cerevisiae*. *Genetics* **147,** 1017-1024.

Holliday, R. (1995). *Understanding Ageing*. Cambridge University Press.

Hu, Z., Yue, Y., Jiang, H., Zhang, B., Sherwood, P. W. and Michels, C. A. (2000). Analysis of the mechanism by which glucose inhibits maltose induction of *MAL* gene expression in *Saccharomyces*. *Genetics* **154,** 121-132.

Ives, P. T. (1950). The importance of mutation rate genes in evolution. *Evolution* **4,** 236-252.

Jazwinski, S. M. and Edelman, G. M. (1984). Evidence for participation of a multiprotein complex in yeast DNA replication in vitro. *Journal of Biological Chemistry* **259,** 6852-6857.

Jelinsky, S. A. and Samson, L. D. (1999). Global response of Saccharomyces cerevisiae to an alkylating agent. *Proceedings of the National Academy of Science U S A* **96,** 1486-1491.

Jiang, R. and Carlson, M. (1997). The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. *Molecular and Cellular Biology* **17,** 2099-2106.

Johnson, F. M. (1999). Carcinogenic chemical-response "fingerprint" for male F344 rats exposed to a series of 195 chemicals: implications for predicting carcinogens with transgenic models. *Environmental and Molecular Mutagenesis* **34,** 234-245.

Johnson, R. E., Washington, M. T., Prakash, S. and Prakash, L. (1999). Bridging the gap: A family of novel DNA polymerases that replicate faulty DNA. *Proceedings of the National Academy of Science USA* **96,** 12224-12226,.

Johnston, M. (1999). Feasting, fasting and fermenting. Glucose sensing in yeast and other cells. *Trends in Genetics* **15,** 29-33.

Jyssum, K. (1968). Mutator factor in *Neisseria meningitidis* associated with increased sensitivity to ultraviolet light and defective transformation. *Journal of Bacteriology* **96,** 165-172.

Keleher, C. A., Redd, M. J., Schultz, J., Carlson, M. and Johnson, A. D. (1992). Ssn6-Tup1 is a general repressor of transcription in yeast. *Cell* **68,** 709-719.

Kendal, W. S. and Frost, P. (1988). Pitfalls and practice of Luria-Delbruck fluctuation analysis: a review. *Cancer Research* **48,** 1060-1065.

Kidwell, M. G., Kidwell, J. F. and Sved, J. A. (1977). Hybrid dysgenesis in *Drosophila melanogaster*: A syndrome of aberrant traits including mutation, sterility, and male recombination. *Genetics* **86,** 813-833.

Kirchman, P. A., Kim, S., Lai, C. Y. and Jazwinski, S. M. (1999). Interorganelle signaling is a determinant of longevity in *Saccharomyces cerevisiae*. *Genetics* **152,** 179-190.

Kishi, T., Seno, T. and Yamao, F. (1998). Grr1 functions in the ubiquitin pathway in Saccharomyces cerevisiae through association with Skp1. *Molecular and General Genetics* **257,** 143-148.

Kopsidas, G. and MacPhee, D. G. (1994). Mutagenesis by 9-aminoacridine in *Salmonella typhimurium*: inhibition by glucose and other PTS class A carbon sources. *Mutation Research* **306,** 111-117.

Kopsidas, G. and MacPhee, D. G. (1996). Frameshift mutagenesis by 9-aminoacridine: antimutagenic effects of adenosine compounds. *Mutation Research* **352,** 135-142.

Koval'tsova, S. V., Gracheva, L. M., Evstiukhina, T. A., Fedorova, I. V., Alekseev, S. I. and Korolev, V. G. (1996). Mutator genes of the yeast *Saccharomyces cerevisiae*: Interaction of mutations *him* and *his* with mutations blocking three principal pathways of repair of induced DNA damage. *Genetika* **32,** 1061-1067.

Kunz, B. A., Ramachandran, K. and Vonarx, E. J. (1998). DNA sequence analysis of spontaneous mutagenesis in *Saccharomyces cerevisiae*. *Genetics* **148,** 1491-1505.

Latarjet, R., Morenne, P. and Berger, R. (1953). *Annales de l'Institut Pasteur* **85,** 174.

Lee, G. S., Savage, E. A., Ritzel, R. G. and von Borstel, R. C. (1988). The base-alteration spectrum of spontaneous and ultraviolet radiation-induced forward mutations in the *URA3* locus of *Saccharomyces cerevisiae*. *Molecular and General Genetics* **214,** 396-404.

Lesage, P., Yang, X. and Carlson, M. (1996). Yeast *SNF1* protein kinase interacts with *SIP4*, a C6 zinc cluster transcriptional activator: a new role for *SNF1* in the glucose response. *Molecular and Cellular Biology* **16,** 1921-1928.

Liefshitz, B., Steinlauf, R., Friedl, A., Eckardt-Schupp, F. and Kupiec, M. (1998). Genetic interactions between mutants of the 'error-prone' repair group of *Saccharomyces*

*cerevisae* and their effect on recombination and mutagenesis. *Mutation Research* **407,** 135-145.

Liviero, L., Wang, Q., Hennig, U. G. G., Ritzel, R. G., Steinberg, C. M. and von Borstel, R. C. (1994). Early and late-arising revertants of the *ura3* allele. *5th Genetics Society of America Conference on Yeast Genetics and Molecular Biology. Seattle*, pp. 195.

Loeb, L. A. and Christians, F. C. (1996). Multiple mutations in human cancers. *Mutation Research* **350,** 279-286.

Lowndes, N. F. and Murguia, J. R. (2000). Sensing and responding to DNA damage. *Current Opinion in Genetics and Development* **10,** 17-25.

Luria, S. and Delbruck, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28,** 491-511.

Lutfiyya, L. L., Iyer, V. R., DeRisi, J., DeVit, M. J., Brown, P. O. and Johnston, M. (1998). Characterization of three related glucose repressors and genes they regulate in *Saccharomyces cerevisiae*. *Genetics* **150,** 1377-1391.

Lutz, W. K. (1990). Endogenous genotoxic agents and processes as a basis of spontaneous carcinogenesis. *Mutation Research* **238,** 287-295.

Lynch, H. T. and de la Chapelle, A. (1999). Genetic susceptibility to non-polyposis colorectal cancer. *Journal of Medical Genetics* **36,** 801-818.

MacPhee, D. G. (1985). Indications that mutagenesis in *Salmonella* may be subject to catabolite repression. *Mutation Research* **151,** 35-41.

MacPhee, D. G. (1993). Directed mutation: Paradigm postponed. *Mutation Research* **285,** 109-116.

Mager, W. H. and Varela, J. C. (1993). Osmostress response of the yeast *Saccharomyces*. *Molecular Microbiology* **10,** 253-258.

Magni, G. E. (1963). The origin of spontaneous mutations during meiosis. *Proceedings of the National Academy of Science USA* **50,** 975-980.

Magni, G. E. (1964). Origin and nature of spontaneous mutations in meiotic organisms. *Journal of Cellular and Comparative Physiology* **64,** 165-172.

Magni, G. E. (1969). Relationships between recombination and mutation in Wolstenholme, G. E. W. and O'Conner, M. (Eds), *CIBA Foundation Symposium on Mutation as Cellular Process*, J. & A. Churchill Ltd, pp. 186-194.

Magni, G. E. and von Borstel, R. C. (1962). Different rates of spontaneous mutation during mitosis and meiosis in yeast. *Genetics* **47,** 1097-1108.

Manivasakam, P., Weber, S. C., McElver, J. and Schiestl, R. H. (1995). Micro-homology mediated PCR targeting in *Saccharomyces cerevisiae*. *Nucleic Acids Research* **23,** 2799-2800.

Marsischky, G. T., Filosi, N., Kane, M. F. and Kolodner, R. (1996). Redundancy of *Saccharomyces cerevisiae MSH3* and *MSH6* in *MSH2*-dependent mismatch repair. *Genes and Development* **10,** 407-420.

McClintock, B. (1951). Chromosome organization and genic expression. *Cold Spring Harbour Symposium of Quantitative Biology* **16,** 13-47.

Mendel, G. (1866). Versuche über Pflanzen-Hybriden. *Verhandlungen des naturforschenden Vereines. Abhandlungen, Brünn*, pp. 3-47.

Mitchel, R. E. and Morrison, D. P. (1983). Heat shock induction of ultraviolet light induction in *Saccharomyces cerevisiae*. *Radiation Research* **96,** 95-96.

Mitchel, R. E. and Morrison, D. P. (1986). Inducible error-prone repair in yeast. Suppression by heat shock. *Mutation Research* **159,** 31-39.

Mohn, G. (1968). Korrelation zwischen verminderter Reparatur-fahigkeit fur UV-Lasionen und hoher Spontanmutabilitat eines Mutatorstammes von E. coli K-12. *Molecular and General Genetics* **101,** 43-50.

Morrison, D. P. and Hastings, P. J. (1979). Characterization of the mutator mutation *mut5-1*. *Molecular and General Genetics* **175,** 57-65.

Muller, H. J. and Altenburg, E. (1919). The rate of change of hereditary factors in *Drosophila*. *Proceedings of the Society of Experimental Biology and Medicine* **17,** 10-14.

Nasim, A. and Brychy, T. (1979). Cross sensitivity of mutator strains to physical and chemical mutagens. *Canadian Journal of Genetics and Cytology* **21,** 129-137.

Neigeborn, L. and Carlson, M. (1987). Mutations causing constitutive invertase expression in yeast: genetic interactions with *snf1* mutations. *Genetics* **115,** 247-253.

Nowell, P. C. (1993). Chromosomes and cancer: the evolution of an idea. *Advances in Cancer Research* **62,** 1-17.

Ozcan, S. and Johnston, M. (1999). Function and regulation of yeast hexose transporters. *Microbiol Mol Biol Rev* **63,** 554-569.

Piper, P. W. (1995). The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiology Letters* **134,** 121-127.

Polaina, J. and Adam, A. C. (1991). A fast procedure for yeast DNA purification. *Nucleic Acids Research* **19,** 5443.

Prakash, L. (1981). Characterization of postreplication repair in *Saccharomyces cerevisiae* and effects of *rad6*, *rad18*, *rev3* and *rad52* mutations. *Molecular and General Genetics* **184,** 471-478.

Prakash, L. and Prakash, S. (1980). Genetic analysis of error-prone repair systems in *Saccharomyces cerevisiae*. in De Serres, F. J., Generoso, W. M. and Shelby, M. D. (Eds), *DNA Repair and Mutagenesis in Eukaryotes*, Plenum Press, pp. 44-78.

Prior, C., Mamessier, P., Fukuhara, H., Chen, X. J. and Wesolowski-Louvel, M. (1993). The hexokinase gene is required for transcriptional regulation of the glucose transporter gene *RAG1* in *Kluyveromyces lactis*. *Molecular and Cellular Biology* **13,** 3882-3889.

Quah, S. K., von Borstel, R. C. and Hastings, P. J. (1980). The origin of spontaneous mutation in *Saccharomyces cerevisiae*. *Genetics* **96,** 819-839.

Ramotar, D., Popoff, S. C., Gralla, E. B. and Demple, B. (1991). Cellular role of yeast Apn1 apurinic endonuclease/3'-diesterase: Repair of oxidative and alkylation DNA damage and control of spontaneous mutation. *Molecular and Cellular Biology* **11,** 4537-4544.

Randerath, E., Watson, W. P., Zhou, G. D., Chang, J. and Randerath, K. (1995). Intensification and depletion of specific bulky renal DNA adducts (I-compounds) following exposure of male F344 rats to the renal carcinogen ferric nitrilotriacetate (Fe-NTA). *Mutation Research* **341,** 265-279.

Randerath, K., Reddy, M. V. and Gupta, R. C. (1981). 32-P Labeling test for DNA damage. *Proceedings of the National Academy of Science USA* **78,** 6126-6129.

Roche, H., Gietz, R. D. and Kunz, B. A. (1994). Specificity of the yeast *rev3* delta antimutator and *REV3* dependency of the mutator resulting from a defect (*rad1* delta) in nucleotide excision repair. *Genetics* **137,** 637-646.

Roche, H., Gietz, R. D. and Kunz, B. A. (1995). Specificities of the *Saccharomyces cerevisiae rad6*, *rad18*, and *rad52* mutators exhibit different degrees of dependence on the *REV3* gene product, a putative nonessential DNA polymerase. *Genetics* **140,** 443-456.

Rossman, T. G. and Goncharova, E. I. (1998). Spontaneous mutagenesis in mammalian cells is caused mainly by oxidative events and can be blocked by antioxidants and metallothionein. *Mutation Research* **402,** 103-110.

Rubin, G. M., Kidwell, M. G. and Bingham, P. M. (1982). The molecular basis of P-M hybrid dysgenesis: the nature of induced mutations. *Cell* **29,** 987-994.

Ruby, S. W. and Szostak, J. W. (1985). Specific *Saccharomyces cerevisiae* genes are expressed in response to DNA-damaging agents. *Molecular and Cellular Biology* **5,** 75-84.

Salz, H. K., Cline, T. W. and Schedl, P. (1987). Functional changes associated with structural alterations induced by mobilization of a P element inserted in the *Sex-lethal* gene of *Drosophila*. *Genetics* **117,** 221-231.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A laboratory manual.*, second edn. Cold Spring Harbour Press.

Sanz, P., Ludin, K. and Carlson, M. (2000). Sip5 interacts with both the Reg1/Glc7 protein phosphatase and the Snf1 protein kinase of *Saccharomyces cerevisiae*. *Genetics* **154,** 99-107.

Sauer, C. O. (1952). *Agricultural origins and dispersals.* MIT Press.

Schaaper, R. M. (1993). Base selection, proofreading, and mismatch repair during DNA replication in *E. coli*. *Journal of Biological Chemistry* **268,** 23762-23765.

Schüller, H. H. and Entian, K. D. (1991). Extragenic suppressors of yeast glucose derepression mutants leading to constitutive synthesis of several glucose-repressible enzymes. *Journal of Bacteriology* **173,** 2045-2052.

Sia, E. A., Jinks-Robertson, S. and Petes, T. D. (1997). Genetic control of microsatellite stability. *Mutation Research* **383,** 61-70.

Soloway, A. H. and Lequesne, P. W. (1980). Potential endogenous mutagens/carcinogens. *Journal of Theoretical Biology* **85,** 153-163.

Spencer, W. P. and Stern, C. (1948). Experiments to test the validity of the linear r-dose/mutation frequency relation in *Drosophila* at low dosage. *Genetics* **33,** 43-74.

Speyer, J. F. (1965). Mutagenic DNA polymerase. *Biochemical and Biophysical Research Communications* **21,** 6-8.

Speyer, J. F., Karam, J. D. and Lenny, A. B. (1966). On the role of DNA polymerase in base selection. *Cold Spring Harbor Symposium on Quantitative Biology* **31,** 693-697.

Stark, M. J. and Milner, J. S. (1989). Cloning and analysis of the *Kluyveromyces lactis TRP1* gene: a chromosomal locus flanked by genes encoding inorganic pyrophosphatase and histone H3. *Yeast* **5,** 35-50.

Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods in Enzymology* **185,** 60-89.

Sturtevant, A. H. (1965). *A History of Genetics*. Harper and Row.

Sutton, W. S. (1902). On the morphology of the chromosome group in *Brachystola magna*. *Biological Bulletin* **4,** 24-39.

Sved, J. (1976). A maternally inherited component in the determination of hybrid dysgenesis in *Drosophila melanogaster*. *Genetics* **83,** s75.

Talpaert-Borlè, M. (1987). Formation, detection and repair of AP sites. *Mutation Research* **181,** 45-56.

Thevelein, J. M. and Hohmann, S. (1995). Trehalose synthase: guard to the gate of glycolysis in yeast? *Trends in Biochemical Sciences* **20,** 3-10.

Tishkoff, D. X., Boerger, A. L., Bertrand, P., Filosi, N., Gaida, G. M., Kane, M. F. and Kolodner, R. D. (1997a). Identification and characterization of *Saccharomyces cerevisiae EXO1*, a gene encoding an exonuclease that interacts with *MSH2*. *Proceedings of the National Academy of Science* **94,** 7487-7492.

Tishkoff, D. X., Filosi, N., Gaida, G. A. and Kolodner, R. D. (1997b). A novel mutation avoidance mechanism dependent on *S. cerevisiae RAD27* is distinct from DNA mismatch repair. *Cell* **88,** 253-263.

Toone, W. M., Aerne, B. L., Morgan, B. A. and Johnston, L. H. (1997). Getting started: regulating the initiation of DNA replication in yeast. *Annual Reviews in Microbiology* **51,** 125-149.

Tran, H., Degtyareva, N., Gordenin, D. and Resnick, M. A. (1997b). Altered replication and inverted repeats induce mismatch repair-independent recombination between highly diverged DNAs in yeast. *Molecular and Cellular Biology* **17,** 1027-1036.

Tran, H. T., Degtyareva, N. P., Koloteva, N. N., Sugin, A., Matsumoto, H., Gordenin, D. A. and Resnick, M. A. (1995). Replication slippage between distant short repeats in *Saccharomyces cerevisiae* depends on the direction of replication and the *RAD50* and *RAD52* genes. *Molecular and Cellular Biology* **15,** 5607-5617.

Tran, H. T., Keen, D. J., Kricker, M., Resnick, M. A. and Gordenin, D. A. (1997a). Hypermutability of homonucleotide runs in mismatch repair and DNA polymerase proofreading yeast mutants. *Molecular and Cellular Biology* **17,** 2859-2865.

Treitel, M. A. and Carlson, M. (1995). Repression by *SSN6-TUP1* is directed by *MIG1* a repressor/activator protein. *Proceedings of the National Academy of Science USA* **92,** 3132-3136.

Trobner, W. and Piechocki, R. (1984a). Selection against hypermutability in *Escherichia coli* during long term evolution. *Molecular and General Genetics* **198,** 177-178.

Trobner, W. and Piechocki, R. (1984b). Competition between isogenic *mutS* and *mut+* populations of *Escherichia coli* K12 in continuously growing cultures. *Molecular and General Genetics* **198,** 175-176.

Trumbly, R. J. (1992). Glucose repression in the yeast *Saccharomyces cerevisiae*. *Molecular Microbiology* **6,** 15-21.

Tsubota, S., Ashburner, M. and Schedl, P. (1985). P-element induced control mutations at the *r* gene of *Drosophila melanogaster*. *Molecular and Cellular Biology* **5,** 2567-2574.

Vincent, O. and Carlson, M. (1998). The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. *European Molecular Biology Organization (EMBO) Journal* **17,** 7002-7008.

von Borstel, R. C. (1966). Effects of radiation on cells. In Schwartz, E. (Ed), *The Biological Basis of Radiation Therapy*, J. P. Lippincott Company, pp. 60-125.

von Borstel, R. C. (1968). On the origin of spontaneous mutations. *XII International Congress of Genetics. Tokyo*, pp. 124-125.

von Borstel, R. C. (1978). Measuring spontaneous mutation rates in yeast. *Methods in Cell Biology* **20,** 1-24.

von Borstel, R. C., Cain, K. T. and Steinberg, C. M. (1971). Inheritance of spontaneous mutability in yeast. *Genetics* **69,** 17-27.

von Borstel, R. C., Graham, D. C., La Brot, K. J. and Resnick, M. A. (1968). Mutator activity of an X-radiation-sensitive yeast. *Genetics* **60,** 233.

von Borstel, R. C. and Higgins, J. A. (1998). Janus Carcinogens and Mutagens. *Mutation Research* **402,** 321-329.

von Borstel, R. C., Ord, R. W., Stewart, S. P., Ritzel, R. G., Lee, G. S., Hennig, U. G. and Savage, E. A. (1993). The mutator *mut7-1* of *Saccharomyces cerevisiae*. *Mutation Research* **289,** 97-106.

von Borstel, R. C., Quah, S.-K., Steinberg, C. M., Flury, F. and Gottleib, D. J. (1973). Mutants of yeast with enhanced mutation rates. *Genetics* **73,** 141-151.

von Borstel, R. C., Savage, E. A., Wang, Q., Hennig, U. G., Ritzel, R. G., Lee, G. S., Hamilton, M. D., Chrenek, M. A., Tomaszewski, R. W., Higgins, J. A., Tenove, C. J., Liviero, L., Hastings, P. J., Korch, C. T. and Steinberg, C. M. (1998). Topical reversion at the *HIS1* locus of *Saccharomyces cerevisiae*. A tale of three mutants. *Genetics* **148,** 1647-1654.

von Tschermak, E. (1900). Über künstliche Kreuzung bei Pisum sativum. *Berichte der deutschen botanischen Gesellschaft* **18,** 232-239.

Vulimiri, S. V., Gupta, S., Smith, C. V., Moorthy, B. and Randerath, K. (1995). Rapid decreases in indigenous covalent DNA modifications (I-compounds) of male Fischer-344 rat liver DNA by diquat treatment. *Chemistry of Biological Interactions* **95,** 1-16.

Wabl, M., Burrows, P. D., von Gabain, A. and Steinberg, C. (1985). Hypermutation at the immunoglobulin heavy chain locus in a pre-B-cell line. *Proceedings of the National Academy of Science U S A* **82,** 479-482.

Wabl, M., Jack, H. M., Meyer, J., Beck-Engeser, G., von Borstel, R. C. and Steinberg, C. M. (1987). Measurements of mutation rates in B lymphocytes. *Immunology Reviews* **96,** 91-107.

Wabl, M. R., Jack, H. M., von Borstel, R. C. and Steinberg, C. M. (1989). Scope of action of the immunoglobulin mutator system. *Genome* **31,** 118-121.

Webster, T. D. and Dickson, R. C. (1983). Direct selection of *Saccharomyces cerevisiae* resistant to the antibiotic G418 following transformation with a DNA vector carrying the kanamycin-resistance gene of Tn*903*. *Gene* **26,** 243-252.

Weinert, T. A. and Hartwell, L. H. (1988). The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science* **241,** 317-322.

Wesolowski-Louvel, M., Goffrini, P., Ferrero, I. and Fukuhara, H. (1992). Glucose transport in the yeast *Kluyveromyces lactis*. I. Properties of an inducible low-affinity glucose transporter gene. *Molecular and General Genetics* **233,** 89-96.

Willems, A. R., Goh, T., Taylor, L., Chernushevich, I., Shevchenko, A. and Tyers, M. (1999). SCF ubiquitin protein ligases and phosphorylation-dependent proteolysis. *Philosophical Transactions of the Royal Society of London Series B Biological Science* **354,** 1533-1550.

Wills, C. (1990). Regulation of sugar and ethanol metabolism in *Saccharomyces cerevisiae*. *Critical Reviews in Biochemistry and Molecular Biology* **25,** 245-280.

Witkin, E. M. (1947). Genetics of resistance to radiation in *Escherichia coli*. *Genetics* **32,** 221-248.

Wolfe, K. H. and Shields, D. C. (1997). Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* **387,** 708-713.

Zakharov, I. A., Kozina, T. N. and Federova, I. V. (1968). Increase of spontaneous mutability in ultraviolet-sensitive yeast mutants. *Doklady (Proceedings) of the Academy of Sciences of the USSR* **181,** 470-472.

Zhou, G. D., Hernandez, N. S., Randerath, E. and Randerath, K. (1999). Acute elevation by short-term dietary restriction or food deprivation of type I I-compound levels in rat liver DNA. *Nutrition and Cancer* **35,** 87-95.

Zimmerman, F. K. and Scheel, I. (1977). Mutants of *Saccharomyces ceerevisiae* resistant to carbon catabolite repression. *Molecular and General Genetics* **154,** 15-82.

## Appendix I.

Sample calculations for the compartmentalization test.

Calculating the spontaneous mutation rate

The compartmentalization method for measuring mutation rates is an extension of the Luria-Delbrück fluctuation test (1943), but it is much simpler, more accurate, and is not plagued with the problem of estimating residual growth of cells. The method, first described by von Borstel, Cain, and Steinberg (1971), was included in a review of a number of ways to measure spontaneous mutation rates (von Borstel, 1978), and now has been used to measure spontaneous mutation rates in mammalian cells (Wabl *et al.*, 1985, 1987, 1989).

Data analysis

1. $N_0 =$ Number of compartments without revertants = $N_t - N_r - N_c$, where $N_t$ is the total number of compartments filled with inoculum, $N_r$ is the number of compartments containing one or more revertant colonies, and $N_c$ is the number of contaminated compartments.

2. $N_0/N = e^{-m} =$ Number of compartments without revertants divided by the number of compartments without contaminants ($N = N_t - N_c$).

3. $\ln e^{-m} = \ln N_0/N = -m$.

4. $(-1)(-m) = m =$ mutational events/compartment = mutational events/ml.

5. $m_g = m - m_b$, where $m_b$ is the number of background mutants ($r$) as calculated from (revertants/ml plated on omission media) x fraction inoculated ($i$) into growth-limiting medium divided by the volume ($v$) of limiting medium to obtain $m_b$/ml.

6. $M = m_g/2C$, where $C$ is the average number of cells/compartment.

7. $M$ = mutations/cell/generation.

Example of data analysis

For demonstration purposes, this example is from an experiment having many background revertants and several contaminated compartments (Expt. 8805-1; von Borstel, personal communication).

1. $N_0$ = 480 compartments - 113 (with colonies) - 11 (contaminated)
   = 356.

2. $N_0/N$ = 356/(480 - 11) = 356/469 = 0.7590618 = $e^{-m}$.

3. ln $e^{-m} = -m$ = -0.275672.

4. $m$ = 0.275672 mutations/compartment
   = 0.275672 mutational events/ml.

5. $m_b$ = (30 + 35 + 36)/ 3 x 0.5 ml/plate) = 67.333333 revertants/ml cell suspension (5 x $10^6$ cells/ml).

   0.25 ml of the cell suspension (inoculum, $i$) is added to the 600 ml (volume, $v$) from which the 480 compartments are filled. Thus, the $i/v$ ratio = 0.25/600 = 0.0004166667.

   And, 67.333333 x 0.0004166667 = 0.280555 background revertants/ml in media added to boxes. 480/600 = 0.8 x 0.0280555 = 0.0224444 = $m_b$.

   $m_g = m - m_b$ = 0.275672 - 0.0224444 = 0.2532276 mutations/ml.

6. $M = m_g/2C$ = 0.2532276 mutations/ml/(2 x 3.0965 x $10^6$ cells/ml).

7. $M$ = 4.08893 x $10^{-8}$ = 4.1 x $10^{-8}$ mutations/cell/generation.

**Appendix 2.**

Comparison of the DNA sequence of *TRP1* and *trp1-11* alleles from *Kluyveromyces lactis*.

The sequence shown is the coding region for the *TRP1* gene from *Kluyveromyces lactis*. The Genbank sequence is derived from Accession #X14230 (Stark and Milner, 1989). JA6 is the parent strain for all of the *K. lactis* strains used in this study and carries the *trp1-11* allele. There is one base difference in the two sequences, located at bp155 that causes the formation of a stop codon (CAA→UAA).

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Genbank GATGCTCGTTAAAGTGTGTGGTTTGCAAACCGTTGAAGCTGCAAAGACTGCTGTGGAT 58
    JA6 -ATGCTCGTTAAAGTGTGTGGTTTGCAAACCGTTGAAGCTGCAAAGACTGCTGTGGAT 57

Genbank GATGGTGCTGATTACTTAGGTATCATTTGTGTTCCCGGTAGGAAAAGAACCATTGACT 116
    JA6 GATGGTGCTGATTACTTAGGTATCATTTGTGTTCCCGGTAGGAAAAGAACCATTGACT 115

Genbank CATCTGTTGCGAAAGGTATTTCAACTGCAGTTCACCAACAAGAGAACGTGAAAGGTAC 174
    JA6 CATCTGTTGCGAAAGGTATTTCAACTGCAGTTCACCAATAAGAGAACGTGAAAGGTAC 173

Genbank TAAACTAGTCGGGGTGTTTAGAAATCAGTCCGTTGATGATGTCCTTCAACTGTACCAC 232
    JA6 TAAACTAGTCGGGGTGTTTAGAAATCAGTCCGTTGATGATGTCCTTCAACTGTACCAC 231

Genbank GAATATAATCTAGATGTGATACAATTACATGGAGATGAAGATATTAAAGAATACAGAT 290
    JA6 GAATATAATCTAGATGTGATACAATTACATGGAGATGAAGATATTAAAGAATACAGAT 289

Genbank CTTTGATCCCATCTTCAATTCCAATCATTAAGAGGTTCCAGTTCCCACAGGATTGTGA 348
    JA6 CTTTGATCCCATCTTCAATTCCAATCATTAAGAGGTTCCAGTTCCCACAGGATTGTGA 347

Genbank ATTACTACTGGACCTGTATGAACACGTAGACAATGTGCTGACGTTGTTCGATTCTGGT 406
    JA6 ATTACTACTGGACCTGTATGAACACGTAGACAATGTGCTGACGTTGTTCGATTCTGGT 405

Genbank GAAGGTGGCACTGGTGAGAAATTGAATTGGAGTGCAATTTCCAGTTGGTCTGCAAGTC 464
    JA6 GAAGGTGGCACTGGTGAGAAATTGAATTGGAGTGCAATTTCCAGTTGGTCTGCAAGTC 463

Genbank ATCCCGAGATAAAATTCATTATCGCTGGTGGATTGAATCCTGATAACGTTTCTGTTGC 522
    JA6 ATCCCGAGATAAAATTCATTATCGCTGGTGGATTGAATCCTGATAACGTTTCTGTTGC 521

Genbank CATTAATATGTTACCAAATGCGATCGGTGTCGATGTAAGTGGAGGAGTAGAGACTGAT 580
    JA6 CATTAATATGTTACCAAATGCGATCGGTGTCGATGTAAGTGGAGGAGTAGAGACTGAT 579

Genbank GGTATCAAGGATTTAGAAAAGGTAAAGCTATTCATCCAGCAGGCCTCTCAATAGAGGA 638
    JA6 GGTATCAAGGATTTAGAAAAGGTAAAGCTATTCATCCAGCAGGCCTCTCAATAGAGGA 637
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Genbank AAAA 642
JA6 AAAA 641